MicroRNA-200b Impacts Breast Cancer Cell Migration and Invasion by Regulating Ezrin-Radixin-Moesin

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Background:
Ezrin-radixin-moesin (ERM) plays an important role in multiple links of tumors. It also involved in breast cancer invasion and metastasis, and might be a potential biomarker of breast cancer. Another study suggested that ERM expression was regulated directly by miR-200c, and had a critical role in miR-200c suppressing cell migration. This study aimed to investigate the effect of miR-200b on ERM expression in a breast cancer cell line and its influence on invasion and metastasis ability in vitro.

Material/Methods:
Breast cancer cell lines MCF-7 and MDA-MB-231 with different metastatic potentials were selected as a model. MiR-200b overexpression or inhibition was achieved by Lipofectamine™ 2000-mediated miRNA transfection. RT-PCR was used to test miR-200b level, while Western blot was selected to detect ERM protein expression. Wound healing assay and Transwell assay were performed to determine cell migration and invasion ability.

Results:
RT-PCR revealed that miR-200b level in MDA-MB-231 was obviously lower than that in MCF-7, while Western blot analysis showed that ERM expression was significantly higher. MiR-200b inhibition by transfection in MCF-7 markedly decreased miR-200b level, elevated ERM expression, and enhanced cell migration and invasion. MiR-200b overexpression in MDA-MB-231 obviously increased miR-200b level, reduced ERM expression, and weakened cell migration and invasion.

Conclusions:
MiR-200b participates in breast cancer cell migration and invasion through regulating ERM in MCF-7 and MDA-MB-231.

MeSH Keywords: Amlodipine • Neuroectodermal Tumors, Primitive, Peripheral • Nuclear Receptor Coactivator 3

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Background

Breast cancer is a common malignant tumor in clinical practice and its incidence is increasing. Early detection and treatment is the only way to improve the survival rate. Breast cancer cells have strong invasive ability and are prone to distant metastases [1], which is a major cause of treatment failure and high mortality [2]. Breast cancer migration and invasion involves the interaction between cancer cell movement ability and host environment. It is a complex non-random process with multiple steps and links [3–5].

MicroRNAs are a popular focus in tumor research [6] and provide a new direction for investigating the mechanism of breast cancer invasion and metastasis. They are common in eukaryotic organisms, with lengths of 17 to 25 nucleotides. MicroRNAs can specifically identify target mRNA and match with 3’-UTR to regulate at the posttranscriptional level. They are involved in various physiological and pathological processes, such as cell proliferation, invasion, and metastasis [7]. It was found that miRNAs are abnormally expressed in a variety of solid cancers and can promote or inhibit tumorigenesis [8]. Recently, the role of the miR-200 family in cancer has been a research focus. Jun discovered that miR-200c inhibiting cell migration [15]. Research showed that ERM plays a key role in tumor invasion and metastasis, and a high level of ERM facilitates tumor invasion and metastasis. Ni et al. found that ERM expression level was positively correlated with breast cancer invasion and metastasis ability [19]. Its invasive ability may be enhanced through epithelial-mesenchymal transformation to promote MMP-9 secretion.

MicroRNAs are a popular topic in tumor research. As a type of small non-coding single-stranded RNA, it exists widely in eukaryotes, with a length of 17 to 25 nucleotides. It is involved in various physiological and pathological processes, such as cell proliferation, invasion, and metastasis [6]. miRNAs are abnormally expressed in a variety of solid cancers and can promote or inhibit tumorigenesis. Recently, the role of the miR-200 family in cancer had been a research focus. Jun discovered that miR-200c expression was positively correlated with E-cadherin in pancreatic cancer specimens, and patients with high miR-200 level had higher survival rates [20]. Bendoraitė et al. found similar results in ovarian cancer [21]. Mny and Sun’s studies revealed that miR-200 expression in epithelial cells can upregulate E-cadherin to reduce tumor migration and invasion through inhibiting transcription factor ZEB1 and ZEB2 expression in epithelial cells [10,11].

Ezrin-radixin-moesin (ERM) plays an important role in the maintenance of cytoskeleton structure and cells movement [12]. As a member of the ERM family, ERM is the connexin between the epithelial cell membrane and cytoskeleton, and has critical effects on cell division, deformation, and movement [13]. Research shows that ERM plays an important role in multiple links of tumor development. It also involved in breast cancer invasion and metastasis and may be a potential biomarker [14]. Previous research showed that ERM expression is directly regulated by miR-200c and plays an important role in miR-200c inhibiting cell migration [15].

Breast cancer has strong invasive ability, which is the major cause of treatment failure and high mortality [2]. Breast cancer metastasis is a non-random, complex process with multi-steps and multi-links, of which cancer cell migration ability is the main influencing factor. Cell actin cytoskeleton plays a key role in migration, and the ERM family has received the most extensive research attention [16]. As a member of the ERM family, ERM is the connection between the epithelial cell membrane and cytoskeleton protein, and is also an important part of the special membrane structure (apical microvilli, plate, or filopodia) [17]. A clinical study suggested that ERM is overexpressed in breast cancer and other tumor cells [18]. Research showed that ERM plays a key role in tumor invasion and metastasis, and a high level of ERM facilitates tumor invasion and metastasis. Ni et al. found that ERM expression level was positively correlated with breast cancer invasion and metastasis ability [19]. Its invasive ability may be enhanced through epithelial-mesenchymal transformation to promote MMP-9 secretion.

Material and Methods

Experimental materials

Breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from Bio-Rad. RPMI 1640, high-glucose DMEM, and fetal bovine serum were purchased from HyClone. Penicillin-streptomycin and trypsin were obtained from Gibco. Lipofectamine™2000 was obtained from Invitrogen. MiR-200b RT-PCR primers, miR-200b mimic and inhibitor, and negative control were from RiboBio. RT-PCR-related reagents (RNA extraction reagent, reverse transcription kit, and SYBR kit) were purchased from TAKARA. Rabbit anti-human ERM primary antibody was from Cell Signaling. β-actin was from Santa Cruz. Matrigel was from BD and Transwell chamber was from Corning Costar.

Cell culture

MCF-7 cells were cultured in RPMI 1640 medium, and MDA-MB-231 cells were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were passaged at 1:2
or 1:3 using trypsin when the fusion reached 90%. The cells in logarithm phase were used for experimentation.

**Cell transfection**

The cells were seeded in 6-well plates and transfected with miR-200b mimic or inhibitor using lipofectamine™2000 according to the manufacturer’s instructions. RNA was extracted at 24 h after transfection. RT-PCR was used to verify miR-200b expression. Wound healing test and Transwell assay were also performed simultaneously. Protein was collected to test ERM expression at 48 h after transfection.

**RT-PCR**

The cells were collected at 24 h after transfection by Trizol. Total RNA was extracted by phenol chloroform method. The purity and concentration were determined by ultraviolet spectrometry. RNA was reverse transcribed to cDNA to perform RT-PCR. The primers sequences were as follows: miR-200b, forward 5’ UAAUACUGCCUGGUAUGUGA3’, reverse 5’ AUCAUUACCAGGCAGUAUAAAU3’; U6, forward 5’ CTGCGTTCGGCAGCACA3’, reverse 5’ AACGCTTCACGAATTTGCGT3’. RT-PCR was performed on Step One real-time PCR amplifier to calculate Ct value and copy number. RT-PCR condition included 95°C for 20 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. U6 was selected as a reference. The results are presented as 2^{–ΔΔCt}.

**Western blot**

Total protein was extracted using RIPA at 48 h after transfection. Its concentration was determined by BCA. Using SDS-PAGE, 20 μg of protein was separated and transferred to an NC membrane. After blocking with 5% skim milk for 2 h, the membrane was incubated with ERM (1:1000) and β-actin (1:2000) primary antibodies at 4°C overnight. Then the membrane was incubated in HRP-tagged secondary antibody (1:5000) at room temperature for 2 h after washing in TBST. ECL was used to develop the protein band. Gel imaging system software was used to analyze band density to calculate ERM relative expression. β-actin was selected as reference.

**Cell migration assay**

Wound healing assay was used to test cell migration. The cells were seeded in 12-well plates and transfected. A 200-μl tip was used to scratch the well. At 0, 12, or 24 h after scratching, cell healing state was observed under an inverted phase contrast microscope (×100). Three random fields of view were selected to test cell migration by measuring the width of scratches by using Image J software. The experiment was repeated 3 times.

**Cell invasion assay**

FBS-free medium was mixed with Matrigel at 3:1. We added 200 μl of mixture to the upper chamber at 37°C for 1 h. We added 200 μl of cells in FBS-free medium containing 0.1% BSA at 1×10^5/mL to the upper chamber, while medium containing

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**Figure 1.** MiR-200b was overexpressed and ERM was decreased in MDA-MB-231 cells compared with MCF-7 cells. (A) miR-200b relative expression level. (B) ERM protein relative expression. ** P<0.01, *** P<0.001, compared with MCF-7.

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10% FBS was added to the lower chamber. After 4-h incubation, the cells were fixed with 70% paraformaldehyde for 15 min and stained by 0.1% crystal violet for 30 min. Six random fields of view were selected under an optical microscope to calculate the cell number. The experiment was repeated 3 times.

**Statistical analysis**

All statistical analysis was performed on SPSS19.0. The data are presented as mean±standard deviation (±s). The t test was used for comparison. *P<0.05, **P<0.01, ***P<0.001, compared with INC.

**Results**

**MiR-200b and ERM expression in breast cancer cell line**

RT-PCR revealed that the miR-200b expression level in MCF-7 (1.01±0.21) was significantly higher than that in MDA-MB-231 (0.17±0.05) (P<0.05, Figure 1A). Western blot analysis showed that the ERM level in MDA-MB-231 (1.87±0.39) was obviously higher than that in MCF-7 (1.03±0.27) (Figure 1B).

**MiR-200b suppression effect in MCF-7**

RT-PCR confirmed that the miR-200b level in MCF-7 was obviously lower after transfection (Figure 2A). Western blot analysis showed that compared with negative control (0.99±0.37), ERM increased markedly after miR-200b inhibition (2.21±0.42)
MCF-7 was further cultured for 12 h and 24 h after miR-200b inhibition. Relative scratch width results showed that it obviously decreased in the miR-200b inhibition group (59.17±5.31, 39.50±6.83)% compared with control (68.33±7.23, 57.18±6.04)%, suggesting that migration ability was enhanced after miR-200b inhibition (Figures 2C, 3). Transwell assay also revealed that after miR-200b inhibition, MCF-7 cell number passing through the membrane at 24 h (131.67±17.41) was significantly higher than that in the negative control (73.01±6.72), indicating that miR-200b inhibition enhanced invasive ability (Figure 2D).

**MiR-200b overexpression impact in MDA-MB-231**

RT-PCR confirmed that miR-200b level in MDA-MB-231 was significantly elevated after transfection (Figure 4A). Western blot analysis revealed that compared with negative control (1.07±0.21), ERM declined markedly after miR-200b overexpression (0.62±0.23) (Figure 4B). MDA-MB-231 was further cultured for 12 h and 24 h after miR-200b overexpression. Relative scratch width results showed that it was obviously increased in the miR-200b overexpression group (70.17±6.51, 59.50±5.24)% compared with control (56.33±8.10, 47.32±6.22)%, indicating that migration ability weakened after miR-200b overexpression (Figures 4C, 3). Transwell assay also showed that after miR-200b overexpression, the number of MDA-MB-231 cells passing through the membrane at 24 h (114.32±7.98) was significantly lower than that in the negative control (114.32±7.98), indicating that miR-200b inhibition enhanced invasive ability (Figure 4D).

**Discussion**

Breast cancer is the most common malignant tumor in females and its incidence rate is rising. We confirmed that the MDA-MB-231 cell line has significantly stronger migration and invasion ability than the MCF-7 cell line by wound healing assay and Transwell assay. Western blot analysis showed that ERM expression in MCF-7 and MDA-MB-231 have different metastasis potentials. ERM expression in highly aggressive MDA-MB-231 was obviously higher than that in MCF-7 with low aggressiveness, consistent with results reported by Carmeci et al. [23]. We also discovered that miR-200b inhibition in MCF-7 led to...
ERM overexpression and promoted cancer cell migration and invasion, further verifying the role of ERM in breast cancer cell migration and invasion. Detecting ERM expression can help to study the biological behavior of breast cancer. We tested miR-200b in MCF-7 and MDA-MB-231 cell lines with different metastatic potentials, and found that its level in MCF-7 was obviously higher than that in MDA-MB-231, showing that miR-200b plays an important role in tumor cell migration and invasion.

We observed the effects of miR-200b inhibition in MCF-7 and miR-200b overexpression in MDA-MB-231. Western blot analysis revealed that miR-200b inhibition in MCF-7 significantly upregulated ERM protein expression, while miR-200b overexpression in MDA-MB-231 clearly reduced ERM level. Wound healing assay and Transwell assay showed that miR-200b inhibition enhanced breast cancer cell migration and invasion, whereas miR-200b overexpression weakened this ability. Our results are similar to those of Li et al. [24], who confirmed that ERM is a target gene of miR-200b on mRNA 3’-UTR. MiR-200b can directly regulate ERM expression and participates in breast cancer metastasis.

Conclusions

This study confirms that miR-200b might be an upstream regulatory factor of ERM. It can regulate ERM expression in breast cancer, thus influencing the biological behavior of breast cancer cells. We hope that our results will provide important assistance in exploring the mechanism of breast cancer migration and invasion, and in developing new treatment methods and improving prognosis.

Figure 4. MiR-200b overexpression suppressed MDA-MB-231 invasion. (A) miR-200b relative expression level. (B) ERM protein expression level. (C) Relative scratch width. (D) Invasive cell number. * P<0.01, *** P<0.001, compared with INC.
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