Journal on Oncology

www.journalononcology.org

Research Article

Open Access, Volume 1

# MiR-4443 Contributes to Metastasis of Breast Cancer Cells Through Suppressing PEBP1

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## Abstract

**Background:** MicroRNAs (miRNAs) involved in every aspect of cancer biology. We aimed to explore the effect of miR-4443 on metastasis of breast cancer (BCa).

**Methods:** Transfection experiment, apoptosis assay, wound healing assay and transwell invasion assay were carried out to assess the influence of miR-4443 on BCa cells. Potential target genes of miR-4443 were predicted and analyzed using bioinformatics method, dual-luciferase reporter assay, and Western blot.

**Results:** MiR-4443 had a higher expression level in highly aggressive MDA-MB-231 cells as compared to poorly aggressive MCF-7 cells. Further study indicated that miR-4443 could increase migration and invasion capability of BCa cells but not apoptotic rate. Dual-luciferase reporter assay indicated that PEBP1 was a target gene of miR-4443. Further study showed that the PEBP1 mRNA and protein expression level was inversely correlated with miR-4443 expression level.

**Conclusions:** In conclusion, our results suggest that miR-4443 contributes to the metastasis of BCa through suppressing PEBP1. Inhibition of miR-4443 in BCa may be a promising therapeutic approach in future.

***Keywords:*** MicroRNA; MiR-4443; PPEBP1; RKIP; Metastasis; Invasion; Biomarker.

**Manuscript Information:** *Received: July 27, 2020; Accepted: August 27, 2020; Published: September 02, 2020*

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**Citation:** *Jin-yan W, Yang Y, Ma Y, Chen Q, Zhang Q, et al. MIR-4443 Contributes to Metastasis of Breast Cancer Cells Through Suppressing PEBP1. J Oncology. 2020; 1(2): 1008.*

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## Introduction

Breast cancer (BCa) is not only the most common cancer but also the first cause of cancer death in women globally [1]. What's more, the incidence and mortality of BCa are increasing rapidly [2]. It was estimated that there were 1,671,149 new cases of BCa and 521,907 cases of deaths due to BCa, worldwide in 2012 [3]. Although early-stage BCa exhibited a favourable prognosis after surgery and chemotherapy, about 90% of BCa deaths were due to the recurrent and distant metastasis of the primary tumor[4]. Hence, identifying novel prognosis and therapeutic biomarkers has become an urgent issue, which may increase the survival rate of these patients.

MicroRNAs (miRNAs) are a class of small, highly conserved and non-coding RNAs, consisting of 18-25 nucleotides. They function as a regulator of gene expression both transcriptional and post-transcriptional levels through binding to the 3'untranslated region (3'UTR) of messenger RNAs (mRNAs) [5]. In the recent decades, accumulating evidences indicated that miRNAs regulate a variety of biological and metabolic process, such as cell apoptosis, proliferation, angiogenesis, drug-resistance, migration and invasion [6-8]. For instance, a number of miRNAs were proved to regulate the invasion and metastasis of tumor [9,10], such as miR-29a [11], miR-34a [12] and miR-105 [13]. While our previous studies have shown that miRNAs could change the drug-resistance of BCa cells [7,14,15], the present study asked if these miRNAs also play a role in promoting invasion and metastasis of BCa. In present study, we focused the effect of miR-4443 which showed a high expression level in drug-resistance breast cells and in patients received preneo-adjuvant chemotherapy [15]. Here, we used highly aggressive MDA-MB-231 cell line and poorly aggressive MCF-7 cell line to explore the modulatory roles of miR-4443 and discover the potential target of miR-4443 involved in the invasion and metastasis of BCa.

## Methods

**Data set of the Cancer Genome Atlas:** Level 3 miRNA-seq isoform quantification (The calculated expression for each individual miRNA sequence isoform observed, per sample) for 1066 BCa tissues and 104 normal breast tissues were downloaded from the data portal for the Cancer Genome Atlas Project (TCGA; accessed November 2017) [16].

**Cell culture:** Human BCa cell line MCF-7 and MDA-MB-231, used in this study, were obtained from the Cell Bank of the Chinese Academy of Sciences (the Cell Bank of the Chinese Academy of Sciences, Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Keygen Biotech, Nanjing, China) high glucose (HyClone), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Carlsbad, CA, America), at 37°C and 5% $CO_2$ in a humidified chamber atmosphere.

**Real-time quantitative PCR (RT-qPCR):** The expression of miR-4443 was detected using stem-loop method as previously described [7] and U6 small nuclear RNA (snRNA) was used as internal control. Regarding to phosphatidylethanolamine-binding protein 1 (PEBP1), β-actin was served as internal control. All the primers used in present study are presented in [Table S1].

Cells in logarithmic phase were collected when a confluence of 80–90% was reached. Total RNAs were isolated using the RNA simple Total RNA Kit (Tiangen, Beijing, China) and reversely transcribed using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, America). The concentration and quality of the RNA were evaluated by the UV absorbance at 260 and 280 nm (260/280 nm, 1.8-2.0) on Nanodrop 2000 spectrophotometry (Thermo Scientific, Carlsbad, America) and by formaldehyde denaturing gel electrophoresis.

RT-qPCR was operated on LightCycler®480 (Roche, Basel, Switzerland), using SYBR Green PCR Master Mix (Roche, Basel, Switzerland) according to the manufacturer's protocol. The Ct values for each gene were normalized to endogenous control, and the relative expression levels were analyzed using the ΔΔCt method and gene expression levels were counted using the $2^{-\Delta\Delta Ct}$ method. Each assay was implemented accompanied with negative controls (nuclease-free water or the extracted RNA without reverse transcription). All sample were detected in triplicate and the data presented implied mean ± SD.

**Cell transfection:** Hsa-miR-4443 mimics, negative control of mimics (mimics-NC); hsa-miR-4443 inhibitors and negative control of inhibitors (inhibitors-NC) were synthesized by Ribobio (Guangzhou, China). The transfectionwas performed as previously described [17]. Briefly, we added miR-4443 mimics or inhibitors or negative controls into $1\times10^6$ cells which were suspend in 100 µl culture medium without fetal bovine serum or antibiotics. The final concentration was 50 nM for mimics and 100 nM for inhibitors. Then, the mixtures were moved into pulse cuvettes, and electroporated by Super Electroporator NEPA 21 Type II (NEPAGENE, Chiba, Japan) (poring pulse: pulse voltage, 125 V; pulse length, 5 ms; pulse interval, 50 ms; pulse number, 2). Blank controls were cells received electroporation without mimicsor inhibitors or their negative controls. After transfection, cells were immediately plated into six-well plate at a density of $5\times10^5$ cells/well with 2 ml of the preheating complete non-antibiotics-culture medium for 24 h.

**Apoptosis assay:** Transfected MCF-7 cells ($5\times10^5$) were seeded in six-well plates with 2 ml of the preheating complete non-antibiotics-culture medium for 24 h. Then, the cells were digested by Pancreatin (Gibco, California, America) without EDTA, washed twice with ice-cold PBS and stained with 5 µl of APC Annexin V and 5ul of 7-AAD (BD Pharmingen, Franklin, America) in the dark for 15 min. Finally, adding 400 µl of 1X binding buffer into each tube and analyzed with the flow cytometer (FACSVerse/Calibur/AriaII-SORP, BD, Franklin, America).

**Wound healing assay:** Transfected BCa Cells were seeded into six-well plate at $5\times10^5$ cells/well with 2ml complete medium for 24h at 37.0$^0$C. After 24h incubation, cell confluence reached about 90% and an artificial scratch wound was created with a 200 µl pipette tip. The suspension cells were dislodged by washing with Phosphate Buffer Saline (PBS) twice. Then, the cells were cultured in serum-free medium. Migration of MDA-MB-231 cells into the wound was verified at 48 h after transfection and MCF-7 cells were observed at 72 h after transfection. Cells that moved

to the wounded area were visualized and photographed under a microscope (Olympus, Tokyo, Japan).

**Transwell Invasion assay:** Matrigel (Corning, Corning, America) diluted 1:9 with antibiotic free medium was equably plated on the membranes (8.0 μm pore size with polycarbonate membrane, Corning, Corning, America) of the upper chamber. After incubation for 2-3 h at $37^0$C, supernatants were removed, $2\times10^4$ BCa cells in 200 μL serum-free medium were seeded to the upper chamber and 500 μL of 25% FBS-containing medium was added to the lower chamber. MDA-MB-231 cells and MCF-7 cells were incubated at $37.0^0$C for 24 h and 48 h, respectively. Then, non-invading cells on the top of the membrane were erased with cotton-tipped swab and invaded cells on the bottom of the membrane were fixed by using 4% formalin and stained with 0.05% crystal violet. Finally, the penetrating cells were qualified by manual enumerating in three randomly selected areas and photographed under the inverted microscope.

**Prediction of miR-4443 target genes:** TargetScan (http://www.targetscan.org; Release 7.1) [18] was used to predict the target gene of miR-4443. To explore the roles of target genes in metastasis, GenClip 2.0 (http://ci.smu.edu.cn/GenCLiP2; Last update: Apr 5, 2016) [19,20] was searched using the predicted genes and the word “metastasis” to hit relative papers, in which “metastasis” and at least one of the genes were appearing together in a sentence.

**Dual-luciferase reporter assay:** The plasmids containing wild-type 3’ UTR of PEBP1 (p-Luc-WT) or mutant-type 3’ UTR of PEBP1 (p-Luc-MT) were constructed using pmiR-RB-REPORT™ vector (RiboBio, Guangzhou, China). Then the constructed plasmids (p-Luc-WT or p-Luc-MT) and miR-4443 mimics (or negative control of mimics) were co-transfected into MCF-7 cells. After transfection, the cells were seeded in 96-well plates (70%–80% confluence). Luciferase activity was determined using the dual luciferase assay system (Promega; Madison, WI) after 24 h of transfection. Luciferase activity was normalized to Renilla luciferase activity.

**Western blot:** Total protein was extracted through blending cells with radio immunoprecipitation assay (RIPA) buffer (Biouniquer Technology, China) on ice for 30 min. Then, the mixture was centrifuged at 14000×g for 15min at 4°C to get rid of cellular debris. The concentration and purity of protein was checked with Nanodrop 2000 spectrophotometry (Thermo Scientific, America). The sample was mixed with sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) buffer (Beyotime, China) and boiled for 5 min. Additionally, equivalent amounts of proteins were subjected to electrophoresis and transferred to polyvinylidene difluoride membranes (PVDF; Sigma-Aldrich, America). After blocking in 5% skim milk in 0.05% Tween20/TBS (TBST) for 1h, the membranes were then probed with the primary antibodies against PEBP1 (1:1000, Abcam, UK) and β-actin (1:6000, Abcam, UK) overnight at 4°C and next incubated with secondary antibodies at room temperature for 1 h. β-actin conducted as an internal loading control to standardize the expression each protein. The secondary antibodies are goat anti-rabbit (1:2000, Abcam, UK) and goat anti-mouse (1:4000, Abcam, UK). After washing with TBST three times, bound proteins were visualized using enhanced chemiluminescence (ECL) plus kit (Biouniquer Technology, China) and captured through the way of film condensation exposure.

**Statistical analysis:** All the statistical analyses were performed using R software (version 3.3.2) and a P value <0.05 was considered statistical significance. All experiments, including invasion assay, RT-qPCR, dual-luciferase reporter assay, western blot, apoptosis assay was independently executed at least three times and the corresponding images listed were representative of three independent experiments. Student’s unpaired t test performed to assess the statistical significance of difference between two independent groups. The data were presented as mean ± SD (standard deviation).

To analyze miRNA-seq isoform quantification data from TCGA, raw counts of miRNAs were normalized and differential miRNA expression was calculated using DESeq2 [21] in the R software.

## Results

**MiR-4443 expression *in vivo*:** We used TCGA data to validate the expression level of miR-4443 *in vivo* and found that miR-4443 had a higher expression level in BCa tissues as compared to normal breast tissues (P<0.01; Figure 1a). The result indicated that miR-4443 may participate in the malignant change of breast cells including invasion and metastasis.

**MiR-4443 expression in human breast cancer cell lines:** We detected the expression of miR-4443 in highly aggressive MDA-MB-231 cells and poorly aggressive MCF-7 cells using RT-qPCR. MiR-4443 expression was 3.00-fold higher in MDA-MB-231 cells than that in MCF-7 cells (P<0.01; Figure 1b). After transfecting miR-4443 mimics or mimics-NC into MCF-7 cells, and transfecting miR-4443 inhibitors or inhibitors-NC into MDA-MB-231 cells, the efficiency of the transfection was examined by qualified the mature miRNA levels by RT-qPCR. MiR-4443 was upregulated by more than 500-fold in cells transfected with miR-4443 mimics compared with those transfected with mimics-NC (P<0.01; Figure 1c). MDA-MB-231 cells transfected with miR-4443 inhibitors had 5.55-fold lower miR-4443 expression level than those transfected with inhibitors -NC (P<0.01; Figure 1d).

**The effect of miR-4443 on BCa cells apoptosis**: Our results indicated that the apoptotic rate of MCF-7 cells transfected by miR-4443 mimics was as same as those transfected with mimics-NC and blank controls (Figure 2a). Similar results were found in MDA-MB-231 cells transfected with miR-4443 inhibitors (Figure 2b), suggesting miR-4443 may have no effect on apoptosis of BCa cells.

**The Influence of miR-4443 on migration and invasionof BCa cells:** To investigate the effects of miR-4443 oninvasion of BCa cells, the wound-healing and transwell invasion assay were further conducted. Wound-healing assay was significantly decelerated in MDA-MB-231 cells transfected with miR-4443 inhibitors, compared with inhibitors-NC control (Figure 3a). Correspondingly, miR-4443 mimics transfected MCF-7 cells showed a greater potential in the process of migration, in comparison with mimics-NC transfected cells (Figure 3b). Similarly, after momentarily transfect-

ing miR-4443 inhibitors, inhibitors-NC or blank control, transwell invasion assay figured that the MDA-MB-231 cells, with the deletion of miR-4443, exhibited poorer invasive capabilities (Figure 3c). Also, MCF-7 emerged a notable faster recovery, when the expression of miR-4443 was reinforced, compared with control cells (Figure 3d), as assessed by transwell invasion assay. Thus, it can be seen that miR-4443 was important not only for BCa cells migration but also for invasion. Collectively, these consequences predicated that miR-4443 validly motivated the migration and invasion of BCa cells, which therefore might lead to the early stages of the malignant progression of BCa. Both wound healing and transwell assay demonstrated that the migration and invasion capability of BCa cells was apparently enhanced with the over-expression of miR-4443 and consistently weakened with the low-expression of miR-4443 *in vitro*.

**Correlation of PEBP1 and miR-4443 in responsible to metastasis of BCa cells:** In order to discover the relationship between miR-4443 and PEBP1, RT-qPCR and Western blot were carried out. As a result, mRNA expression of PEBP1 was 5.56-fold lower in MDA-MB-231 cells than that in MCF-7 cells, which was also proved by western blot (Figure 4b). Actually, MCF-7 cells transfected with miR-4443 mimics had 4.17-fold lower PEBP1 mRNA expression level than those transfected with mimics-NC, and the expression of PEBP1 protein also showed similar result (Figure 4c). The same tendency was seen in MDA-MB-231 cells transfected with miR-4443 inhibitors compared with inhibitors-NC (Figure 4d). Taking together, the correlation between miR-4443 and PEBP1 illustrated that PEBP1 was a target of miR-4443, through which miR-4443 may increase the invasion and metastasis of BCa cells.

## Discussion

In the past decade, growing evidence has indicated that abnormal expression of certain miRNA is directly associated with tumor invasion and metastasis [22] through base-pairing with the 3'- UTRs of mRNA [23]. For example, miR-200 inhibited the metastasis of lung adenocarcinoma though targeting Extra-Cellular Matrix (ECM) proteins and peptidases [24]. MiR-19 contributed to invasion and metastasis of advanced Colorectal Cancer (CRC) via targeting transglutaminase-2 (TG2) [25]. Recently, PEBP1 (also known as RKIP, for Raf kinase inhibitor protein) [26], had been widely recognized as a novel and clinically relevant metastasis suppressor genes [27]. PEBP1 took part in the Raf-1–mediated phosphorylation and the activation of MEK, and involved in the regulation of a variety of vital cellular processes, including proliferation, differentiation, survival, and cell death [28]. The expression of PEBP1 was frequently downregulated in various aggressive cancers, such as BCa [29], gastric cardia adenocarcinoma (GCA) [30], hepatocellular carcinoma (HCC) [31], non-small cell lung cancers (NSCLC) [32], CRC [33] and so on. Furthermore, PEBP1 was frequently reported to suppress the invasion and metastasis of BCa by regulating matrix metallopeptidase 13 (MMP13) [34], Chemokine (C-C motif) ligand 5 (CCL5) [35], High-mobility group AT-hook 2 (HMGA2) [36] and stroma-associated genes [37] and so on. In our actual research, we discovered miR-4443 was upregulated in highly invasive MDA-MB-231 cells, compared to nonmetastatic MCF-7 cells. The result suggests that miR-4443 may be a potential onco-miRNA, facilitating the invasion and metastasis of BCa. To affirm this notion, we certified that ectopic expression of miR-4443 strengthened the invasion and metastasis of BCa through wound healing assay and Transwell Invasion assay. Moreover, upregulation of miR-4443 could increase the apoptotic rate. To understand the molecular mechanisms of miR-4443 enhancing the BCa cells, we identified the PEBP1 as a target of miR-4443 us-

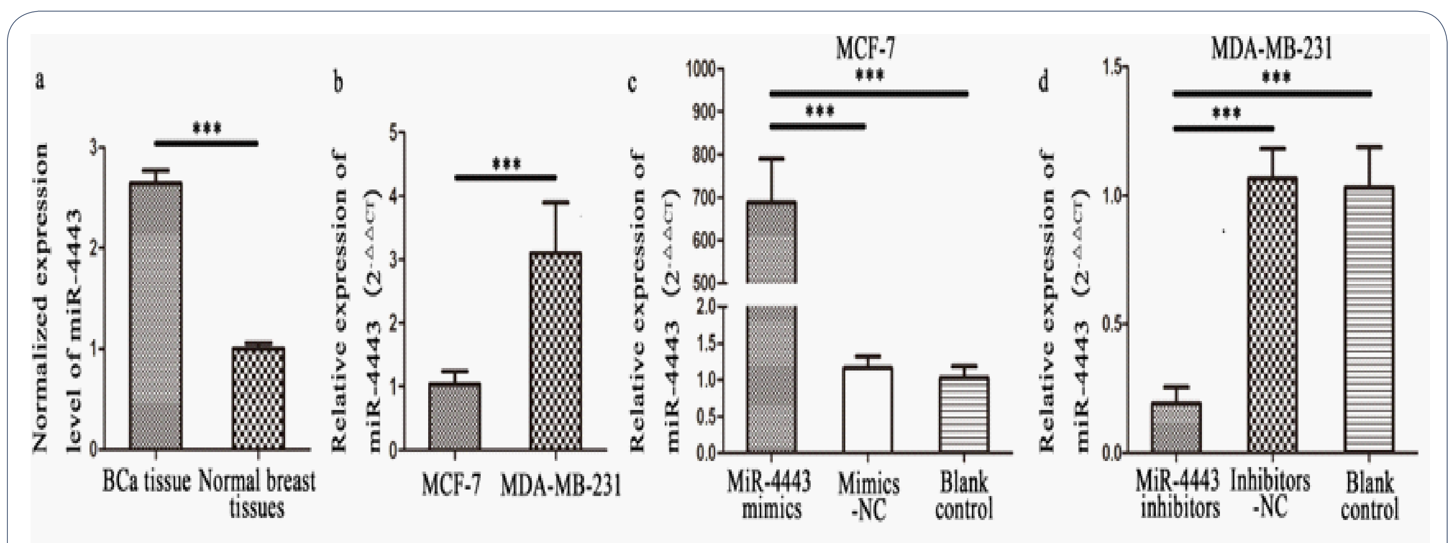


**Figure 1:** Quantification of miR-4443 in different BCa cells.
a: Quantification of miR-4443 in BCa tissue and normal breast tissue. b:Quantification of miR-4443 in MDA-MB-231 and MCF-7 cell lines. c: Quantification of miR-4443 in MCF-7 cells transfected with miR-4443 mimics compared with blank control and mimics-NC. d: Quantification of miR-4443 in MDA-MB- cells transfected with miR-4443 inhibitors compared with blank control and inhibitors-NC. Bars display as the mean ± SD from three independent experiments. **P <0.01 or ***P<O.001, compared with the blank control and NC.

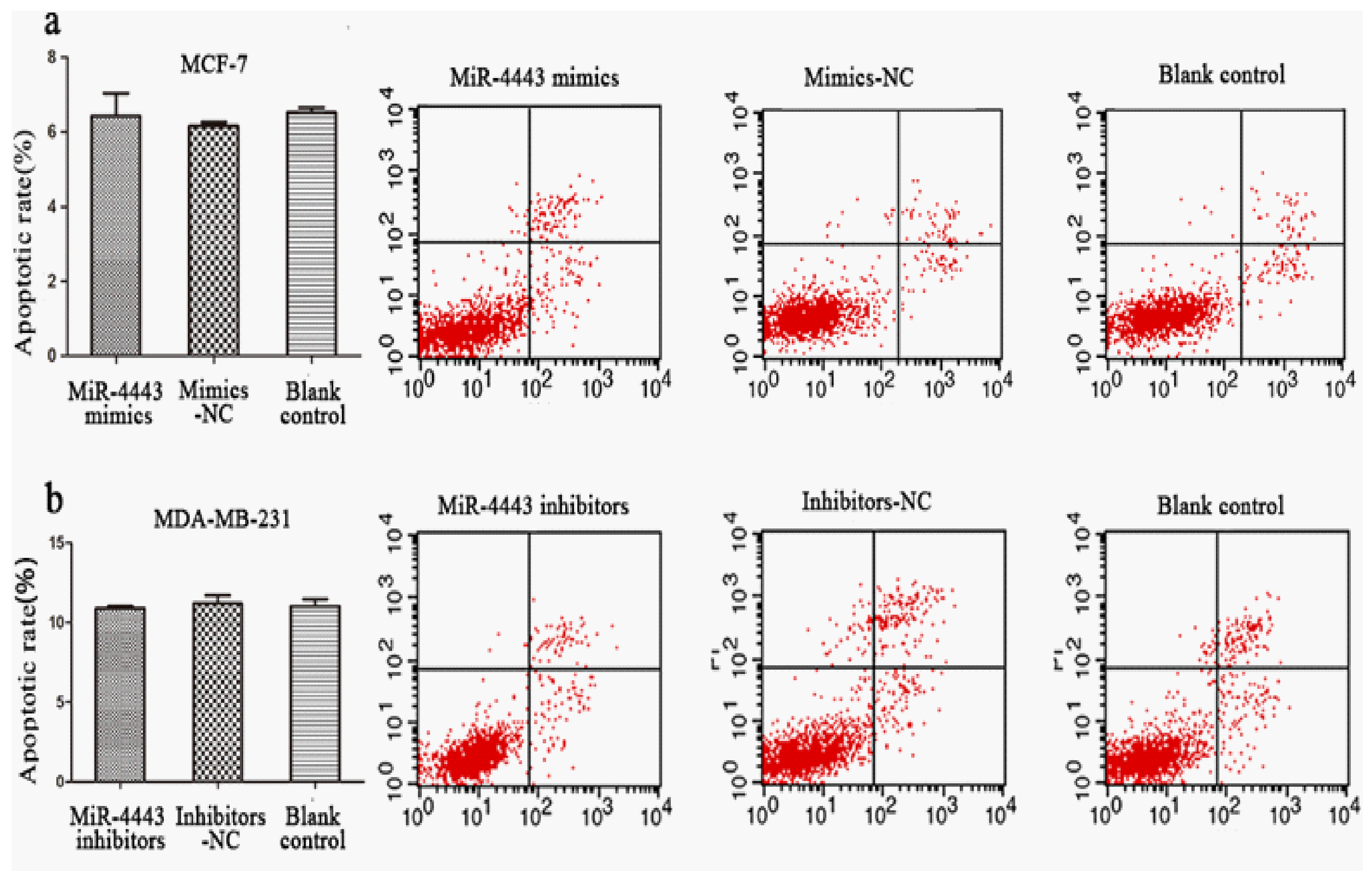


**Figure 2:** Flow cytometry assessment of apoptotic MCF-7 and MDA-MB-231 cells induced by miR-4443.
a: The apoptotic rate of MCF-7 cells transfected with miR-4443 mimics was as same as those transfected with mimics-NC and blank controls. b: The apoptotic rate of MDA-MB-231 cells transfected with miR-4443 inhibitors was as same as those transfected with inhibitors-NC and blank controls. Bars indicate the mean ± SD from at least three independent experiments, compared with the blank control and NC. The corresponding flow cytometry images were also shown.

ing bioinformatic analysis and dual-luciferase reporter assay, and confirmed that miR-4443 could restrain the endogenous expression level of PEBP1 using RT-qPCR and Western Blot.

However, not only the mechanism of the high expression of miR-4443, but also the role of miR-4443 in BCa progression remains indistinct. Meerson A et al claimed that the up-regulation of miR-4443 in CRC was connected with high doses of leptin and insulin [38]. No more findings revealed the mechanism of miR-4443 up-regulation, thus further research is necessary to detect the way of regulating miR-4443 expression in BCa. At the same time, further efforts are also required to verify other underlying target genes of miR-4443 and validate the pivotal pathways in suppressing BCa progression. Taken together, understanding the proper role of miR-4443 in BCa progression will not only aggrandize our knowledge of BCa biology but may also confirm miR-4443 as a novel target for the BCa therapy and prognostic indicator.

In conclusion, the vital discovery of our present study is that the upregulation of miR-4443 increases BCa cell invasion by suppressing PEBP1. We have therefore shown that miR-4443 plays an essential role in regulating BCa cell invasion and metastasis and may function as an onco-miRNA.

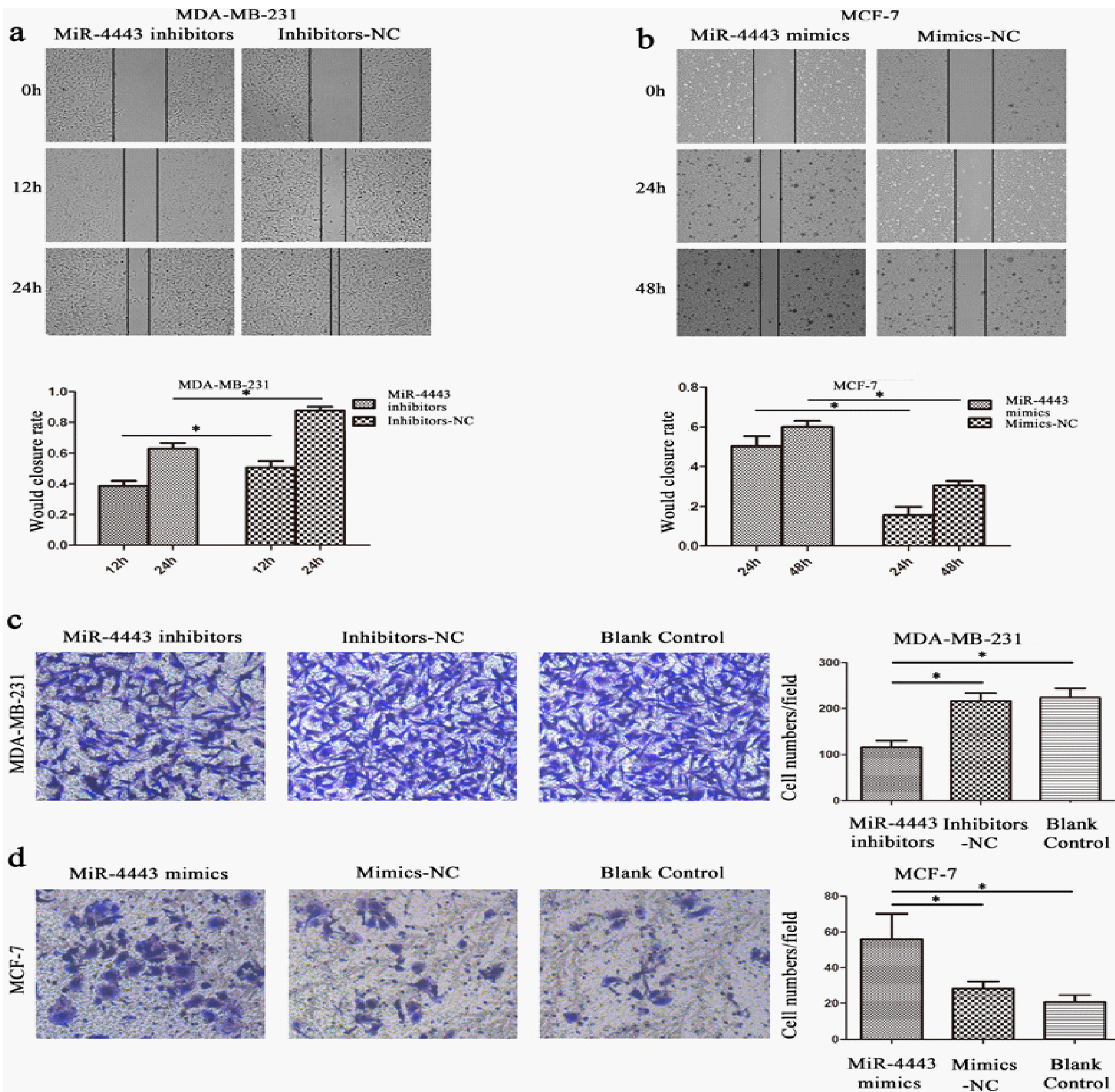


**Figure 3:** Effect of miR-4443 on the invasion and metastasis ability of BCa cell lines.
a: The wound healing assay was performed in MDA-MB-231 cells transfected with miR-4443 inhibitors, compared with inhibitors-NC and blank control. b: The wound healing assay was performed in MCF-7 cells transfected with miR-4443 mimics, compared with mimics-NC and blank control. c: The cell invasion assay was performed in MDA-MB-231 cells transfected with miR-4443 inhibitors compared with inhibitors-NC and blank control. d: The cell invasion assay was performed in MCF-7 cells transfected with miR-4443 mimics compared with mimics-NC and blank control. Data are shown as mean ±SD; *P<0.05.

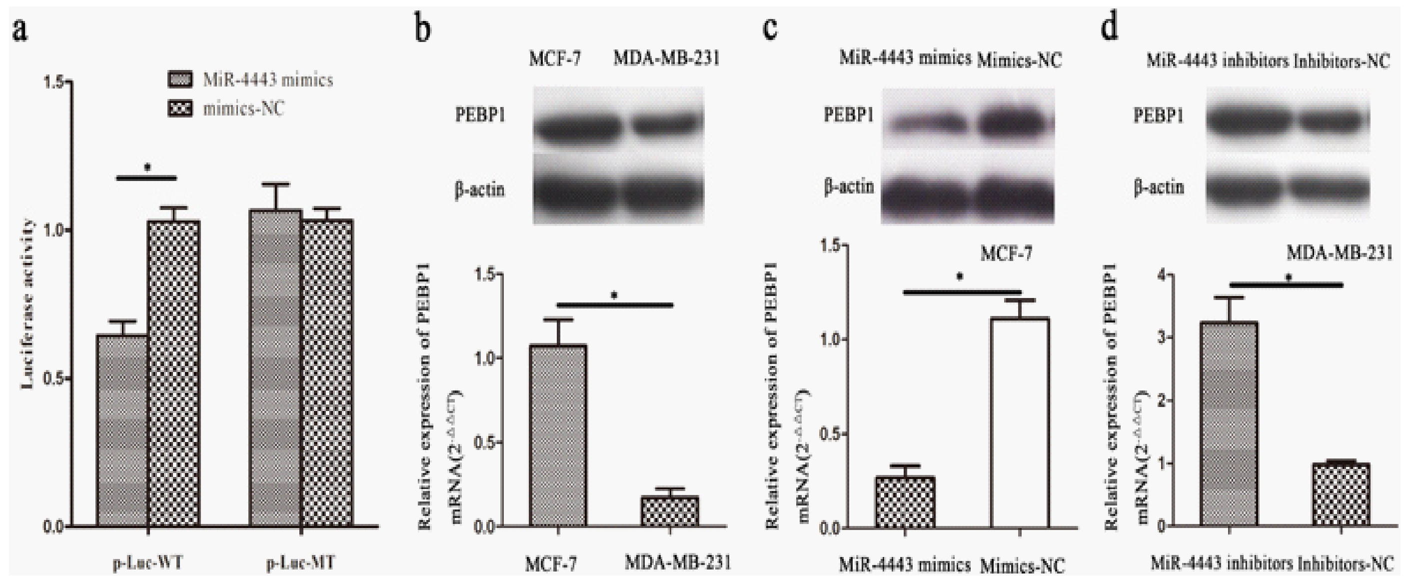


**Figure 4:** RT-qPCR and Western blot analysis was performed in MCF-7 and MDA-MB-231 cells.
a: Luciferase assay of MCF-7 cells co-transfected with MiR-4443 mimics and plasmids. mimics-NC, negative control of mimics; p-Luc-WT, the plasmids containing wild-type 3' UTR of PEBP1; p-Luc-MT, the plasmids containing mutant-type 3' UTR of PEBP1. c: RT-qPCR and western blot results of PEBP1 expression in MCF-7 cells transfected with miR-4443 mimics and mimics-NC. d. RT-qPCR and western blot results of PEBP1 expression in MDA-MB-231 cells transfected with miR-4443 inhibitors and inhibitors-NC. Data are shown as mean ±SD; *P<0.05.

**Table 1:** The top 20 genes by co-searching GenClip 2.0 using target genes of miR-4443 and the term "metastasis".

| Gene[1] | Hit[2] | Total[3] | Hit/Total |
|---|---|---|---|
| PEBP1 | 127 | 676 | 0.188 |
| FLT4 | 148 | 1665 | 0.089 |
| CDH1 | 1084 | 14418 | 0.075 |
| MMP2 | 926 | 16803 | 0.055 |
| CCR7 | 121 | 2631 | 0.046 |
| PTK2 | 222 | 5136 | 0.043 |
| HGF | 338 | 8386 | 0.04 |
| TIMP2 | 153 | 4306 | 0.036 |
| CDH2 | 120 | 3457 | 0.035 |
| CAV1 | 150 | 4325 | 0.035 |
| HIF1A | 268 | 8021 | 0.033 |
| EGFR | 832 | 36142 | 0.023 |
| PGR | 278 | 13480 | 0.021 |
| CTSB | 126 | 8147 | 0.015 |
| TG | 116 | 8779 | 0.013 |
| AKT1 | 516 | 44234 | 0.012 |
| EGF | 152 | 19511 | 0.008 |
| BCL2 | 249 | 40622 | 0.006 |
| MAPK1 | 291 | 53520 | 0.005 |
| MAPK3 | 107 | 23983 | 0.004 |

[1] The listed genes are those found in the sentences identified by the term "metastasis".

[2] The number is the count of articles mentioning the corresponding gene and "metastasis" in one sentence.

[3] The number is the count of articles mentioning the corresponding gene.

**Acknowledgements**

This study was funded by the National Natural Science Foundation of China (grant number 81602551, 81702613), Key Research Program for the Social Development of Jiangsu Province, China (BE2015718), National Program on Key Research Project (No. 2016YFC0905900), Medical Training Programme Foundation for the Talents by Jiangsu Provincial Department of health (No.17 [2016]), The 333 Talent Project of Jiangsu Province, top-level (No.4 [2016]), the National key clinical specialist construction Programs of China (No. 544 [2013]), Major Program of Natural Science Foundation of Jiangsu Province (No. BL2014090), Natural Science Foundation of Jiangsu Province (No. BK20151579), A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, JX10231802), Postgraduate Research & Practice Innovation Program of Jiangsu Province (SJCX17_0387), the young talents program of Jiangsu Cancer Hospital (2017YQL-10).

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