

ORIGINAL ARTICLE

***In vivo* and *in vitro* impact of miR-31 and miR-143 on the suppression of metastasis and invasion in breast cancer**

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Summary

Purpose: The microRNA (miR)-31 and miR-143 are pleiotropic anti-metastatic miRs, with an expression that decreases significantly in metastatic breast cancer cells. The aim of this study was to investigate the effect of miR-31 and miR-143 inhibition on metastasis and invasion in both MDA-MB231, MDA-MB468 as well as the MCF-7 breast cancer cell lines and 5-week old female mice.

Methods: Following the cloning of miR-31 and miR-143 into vectors, their expressions were determined before treatment with constructs of miR-31 and miR-143 in cancer cell lines and normal breast cells. Then miR-31 and miR-143 were transfected to the cell lines and the expression was assessed after 48 hrs. Moreover, the levels of migration and invasion were determined in cell lines. These experiments were performed in 5-week old female mice.

Results: The results showed that miR-31 expression before

the transfection of miR-31 construct was decreased 4, 70 and 100 times in MCF-7, MDA-MB468 and MDA-MB231 cell lines, respectively, in comparison to normal breast cells; but after the transfection of miR-31 construct, the expression of miR-31 increased 80 times. Additionally, invasion and migration decreased by 15 and 10 times in MDAMB-468. All of the modifications in miR-143 were low in comparison to miR-31. The results of the *in vivo* experiments were approximately the same as in the *in vitro* experiments.

Conclusions: It appears that the use of miR-31 is highly efficient than miR-143 in the inhibition of invasion and metastasis in breast cancer. Our study improved our conception about miR-31 and miR-143 and their roles in the identification and therapy of breast cancer.

Key words: breast cancer, invasion, metastasis, microRNA

Introduction

Breast cancer (BC) is the most common cancer in women and the leading cause of cancer-related deaths in females worldwide [1]. Due to recent de-

velopments, the mortality of BC decreased, however it is estimated that about 1.3 million women are affected by BC every year [2,3]. The current

treatment methods include chemotherapy, surgery, radiotherapy, hormonal treatment and targeted therapy [4]. Over the past decade, the studies largely focused on miRs as new biomarkers in the diagnosis and treatment of BC [5,6].

Metastasis is a process in which a tumor cell leaves the primary tumor, travels to a distant site via the circulatory system and establishes a secondary tumor [7]. Tumor metastasis is a key event in the progression of BC and is mainly responsible for BC-associated mortality [8].

miRs are small noncoding RNAs, which are encoded in the genome of many species. These molecules are involved in translation, RNA stability and gene expression [9]. Increasing evidence indicates that dysfunction of miRs are involved in the development of cancer, suggesting that miRs can function as classical oncogenes or tumor suppressor genes [10]. The expression of miRs exerts an effect on processes associated with cancer progression, such as invasion, metastasis, and apoptosis [11]. Many studies demonstrated that miRs play important role in tumor initiation by regulation of tumorigenicity, self-renewal ability and drug resistance in cancer stem cells [12].

miR-31 plays an important role in different types of cancers, such as BC [13,14], ovarian cancer [15,16], lung cancer [17,18], colon cancer [19,20] and melanoma [21,22]. Also, miR-143 was identified as one of the low-expressed miRs in various tumors, including non-small cell lung cancer [23], gastric cancer [24], colorectal cancer [25], pancreatic cancer [26], cervical cancer [27], prostate cancer [28], osteosarcoma [29] and leukemia [30]. The expression of miRs is changing in various types of cancers and act as tumor suppressive or oncogenic factors, depending on the type of cancer [31].

The aim of this study was to investigate the effects of miR-31 and miR-143 on the cell invasion and metastasis processes in MDA-MB231, MDA-MB468 and MCF-7 breast cancer cell lines (*in vitro*) and 5-week old female mice (*in vivo*).

Methods

Cell culture and preparation

Three breast cancer cell lines including MDA-MB231, MDA-MB468 and MCF-7 were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (200µg/ml). Normal breast cells were taken from healthy women and washed three times by sterile phosphate buffered saline (PBS) to remove blood and cells. Then, samples were cut into small pieces and incubated in dissociation buffer DMEM medium supplemented with 2% FBS 2 plus antibiotic solution, collagenase type I

(Worthington Biochemical Corporation, Lakewood/USA), hyaluronidase (Sigma Aldrich, Saint Louis/USA) for 24 hrs at 37°C. Then, the cells were centrifuged and supernatants were stored in pellet. Three ml warm Trypsin/EDTA were added and re-suspended in the pellet. Ten ml of Hank's buffer and 2% cold FBS were added to neutralize trypsin and centrifuged for 5 min. Warm Dispose 2Ml (Stemcell Technologies, Vancouver/Canada, 5mg/ml) and 200µl DNase I (Thermo Fisher Scientific, Waltham/USA, 1mg/ml) were added to the pellet and re-suspended for 1 min. The suspension was diluted with 10ml cold Hank's buffer solution and passed through 40-µm cell strainer. The suspension was centrifuged for 5 min and 1 ml medium was added to the pellet and cell counting was performed.

Cloning by vector

The miR-31-mimic and miR-143-mimic oligomers were designed and synthesized according to pcDNA 6.2gw/EmGFP vector kits protocol. The double-stranded oligomers hybridization was performed according to manufacturer's instructions. Electrophoresis was performed as follows: the ligation of double-strands fragments to the vectors was performed with 5× ligation buffer, pcDNA 6.2gw/EmGFP vector (5ng/µl), double-stranded oligo (10nM), nuclease free water, T4DNA ligase enzymes (1U/µl), and transmission to the bacteria was implemented through electric shock. Cloning accuracy was performed with Clooney-PCR by specific primers vector and the results were analyzed using electrophoresis.

Transfection and flow cytometry

5×10⁴ cells were cultured from each cell line in 24 plates with 60% confluence. The cells were transfected with lipofectamine and two pcDNA 6.2gw/EmGFP vectors (Promega, Madison, Wisconsin, USA) with miR-31 and without miR-31 (positive control). Forty-eight hrs after transfection, cells were dislodged using 0.25% trypsin and green fluorescent protein (GFP) expression was assessed by flow cytometry to determine the rate of transfection. Extraction of miR from three cell lines and normal breast cells was performed before and after treatment with the vector. Then cDNA was synthesized.

Real time PCR

Real time PCR was performed to analyze the miR-31 and miR-143 expression levels, by Rotor-Gene 3000 kits and primer. After determining the transfection rate, three cells lines were classified in three groups, such as control, with and without miR and were used to create the scratch test.

We used the SYBR Premix ExTaq (Takara, Mountain View/USA) with the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA). The real time PCR reaction included initial denaturation at 95°C for 10 min, denaturation at 95°C for 10s (40 cycles), annealing at 55°C for 30s, and extension at 72°C for 60s, respectively. Finally, changes of mRNA expression were evaluated by 2^{-ΔΔCt} method.

Migration and invasion assays

Evaluation of invasion and migration was performed by Matrigel-coated filters with 0.8 micron pore size. Cells were placed on the filters, incubated for 24 hrs and removed from the culture medium. The filters' bottom were then fixed in paraformaldehyde (PFA) and stained with crystal violet. In the following step, 10 random pictures were taken from each one of the wells. In order to calculate invasion and migration, cell counting was performed in two conditions (with Matrigel and FBS and without Matrigel).

Animal studies

The MDA-MB231, MDAMB-468 and MCF-7 cells were infected with pLV3 or pLV3-720 and suspended in PBS. BALB/c nude female mice (20 control and 20 case) aged 4 to 5 weeks were used in this study. For the experiments, mice were implanted with 5×10^5 cells via intravenous tail injection. Six weeks later, mice were sacrificed. The breasts of the mice were fixed and stained with hematoxylin and eosin. Breast metastasis was quantified by counting the number of tumor foci in 10 randomly selected high-power fields.

Statistics

Data from at least three independent experiments are presented as mean \pm SD. The *t*-test was used for comparisons between groups unless otherwise noted. Data comparisons used paired *t*-test and group comparisons used one-way analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant.

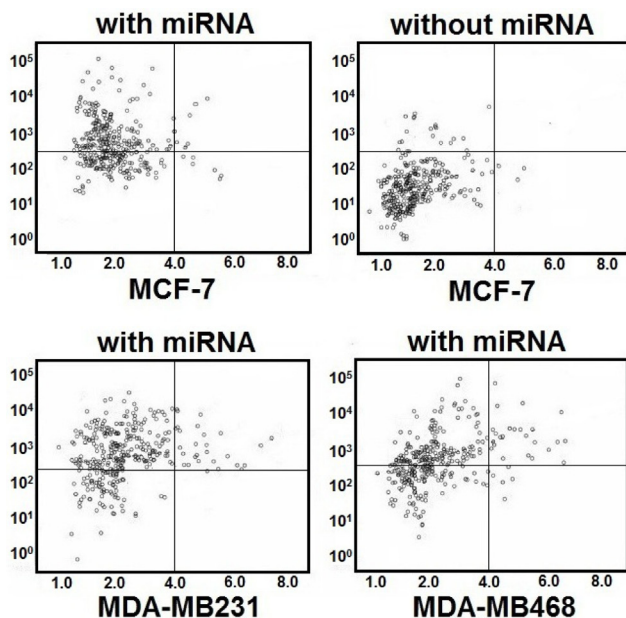


Figure 1. The cells were transfected by vector with miRs and without miRs and assessed by flow cytometry to determine the rate of transfection. The results showed that the rates of transfection in MCF-7 cells were 26.1% (with miR) and 35.3% (without miR), in MDA-MB231 cells were 28.3% (with miR) and 33.7% (without miR) and in MDA-MB-468 were 26.8% (with miR) and 33.2% (without miR).

Results

Results of in vitro condition

Double-stranded hybridization was confirmed by electrophoresis. After cloning, an appropriate colony was selected by colony-PCR method and the obtained plasmids were purified. The MDA-MB231, MDA-MB468 and MCF-7 cell lines were infected with vectors without miRs and with miRs and flow cytometrically analyzed (Figure 1).

The real time PCR was performed with LNA miR primers to analyze the expression rates. The obtained results before transfection of miR-31 construct showed that the miR-31 expression was decreased 4, 70 and 100 times in MCF-7, MDA-MB468 and MDA-MB231, respectively, as compared to normal breast cells. Also, the expression analysis after treatment of cells with vectors with miR-31 and without miR-31 revealed that the expression of miR-31 increased 80 times in MDA-MB231 cell line (Figure 2).

To determine the potential role of miR-31 and miR-143 in breast cancer, invasion and migration assays were performed. The invasion was reduced 15 and 10 times in the MDA-MB231 and MDAMB-468 cell lines, respectively. On the other hand, invasion was reduced insignificantly in the MCF-7 cell line (Figures 3 and 4).

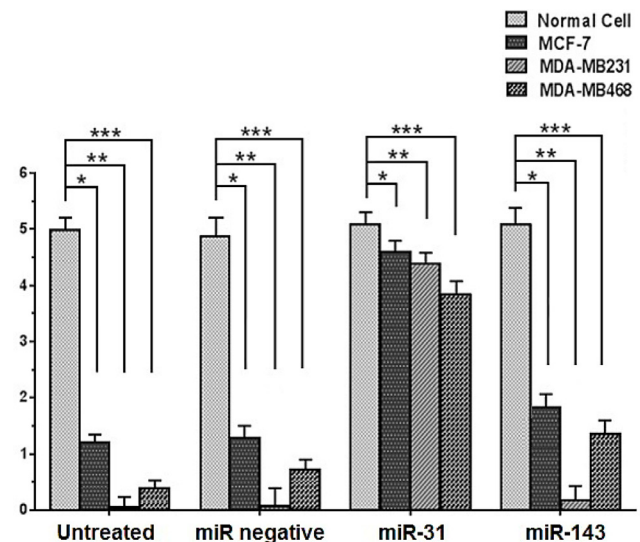


Figure 2. The expression of miR-31 and miR-143 were compared in MDA-MB231, MDAMB-468, MCF-7 and control cells. Untreated cell lines ($*p=0.002$, $**p=0.001$, $***p=0.0022$); miR negative ($*p=0.013$, $**p=0.0012$, $***p=0.0006$); miR-31 ($*p=0.0032$, $**p=0.0043$, $***p=0.0012$); miR-143 ($*p=0.09$, $**p=0.055$, $***p=0.08$). The obtained results showed that after treatment of cells with vectors with miR-31 significantly increased the expression of miR-31 in breast cancer cell lines.

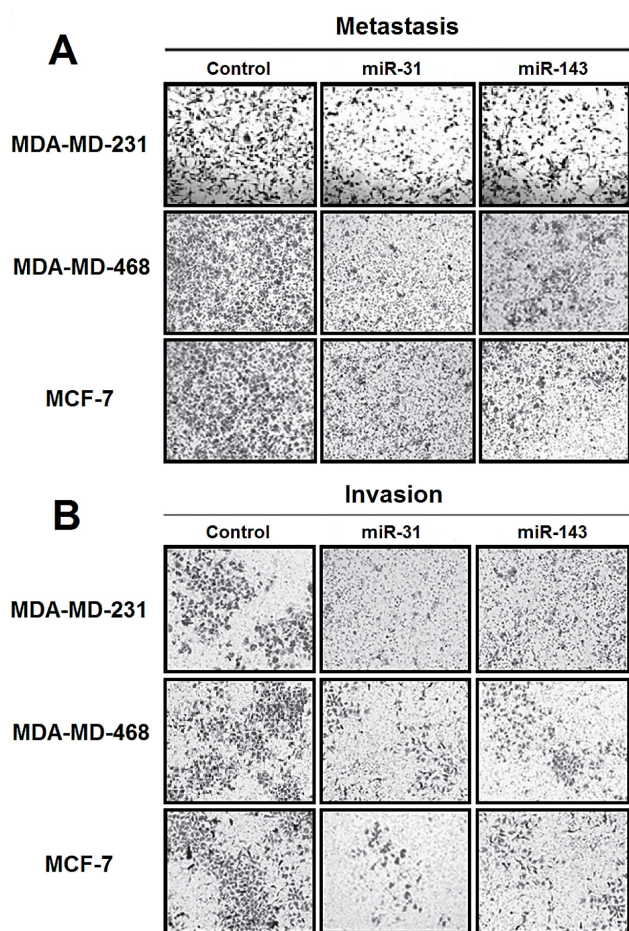


Figure 3. Effects of miR-31 and miR-141 mimics transfection on invasion and migration of MDA-MB231, MDA-MB468 and MCF-7 cell lines. The miR-31 and miR-143 decreased metastasis (A) and invasion (B) in MDA-MB231, MDA-MB468 and MCF-7 cells. The matrigel assay showed that the rate of invasion and metastasis was significantly lower in the miR-31-transfected cells than the control group ($p < 0.05$).

The obtained results before transfection of miR-143 construct showed that the miR-143 expression was decreased 2, 10 and 15 times in MCF-7, MDA-MB468 and MDA-MB231, respectively, as compared to normal breast cells. Also, expression analysis after treatment of cells with vectors with miR-143 and without miR-143 showed that the expression of miR-143 increased insignificantly in the cell lines. The invasion was reduced 4, 3 and 4 times in the MDA-MB231, MDAMB-468 and MCF-7 cell lines, respectively (Figures 3 and 5).

Results of in vivo condition

To confirm that the expression of miRs suppresses migration and invasion, nude mice were injected with MDA-MB231, MDAMB-468 and MCF-7 cells expressing miR-31 and miR-143 via intravenous tail injection. Six weeks post-injection, body weights of the mice did not differ. However, the obtained results from miR-31 groups were significantly lighter as compared with the control group. On the other hand, only 7 out of the 20 mice (35%) from the miR-31 group showed metastasis. In the control group, 14 out of the 20 mice (70%) developed breast metastasis. Also, the obtained results from miR-143 group were not significantly different as compared to the control group. In this group, 13 out of the 20 mice (65%) from the miR-143 group developed breast metastasis (Figure 6).

Discussion

In this study we investigated the expression of miR-31 and miR-143 in breast cancer progression in the three breast cancer cell lines. In over

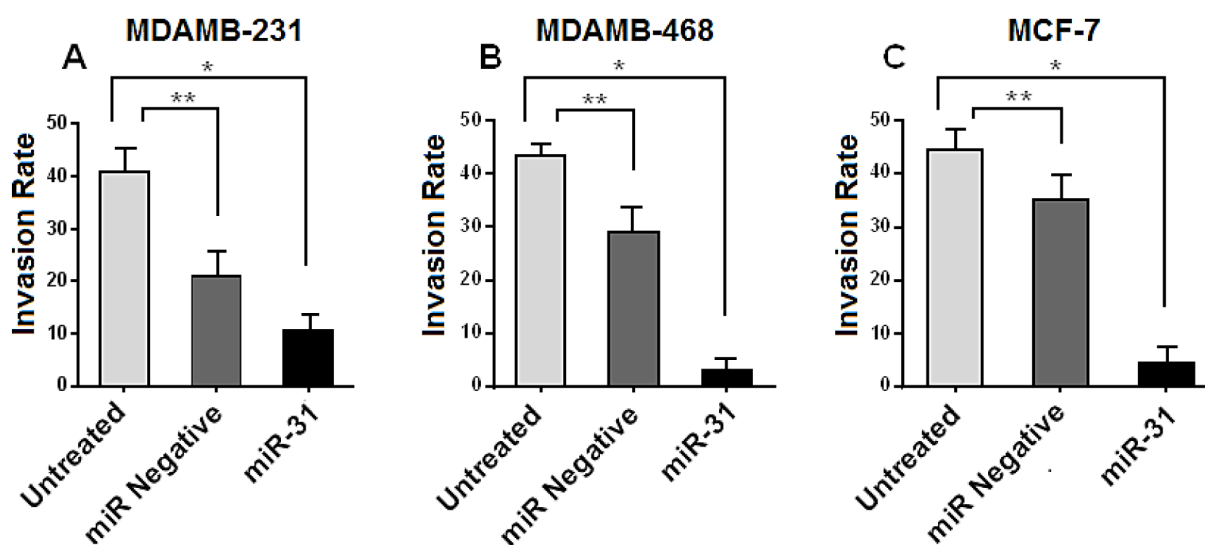


Figure 4. Cell invasion rate in untreated, treated without miR-31 and treated with miR-31 in MDAMB-231 (A), MDAMB-468 (B) and MCF-7 (C) cell lines (*in vitro*). The miR-31 was significantly downregulated in MDAMB-231, MDAMB-468 and MCF-7 cells. (A) $*p = 0.023$ and $**p = 0.01$; (B) $*p = 0.0037$ and $**p = 0.0031$; (C) $*p = 0.0024$ and $**p = 0.0012$.

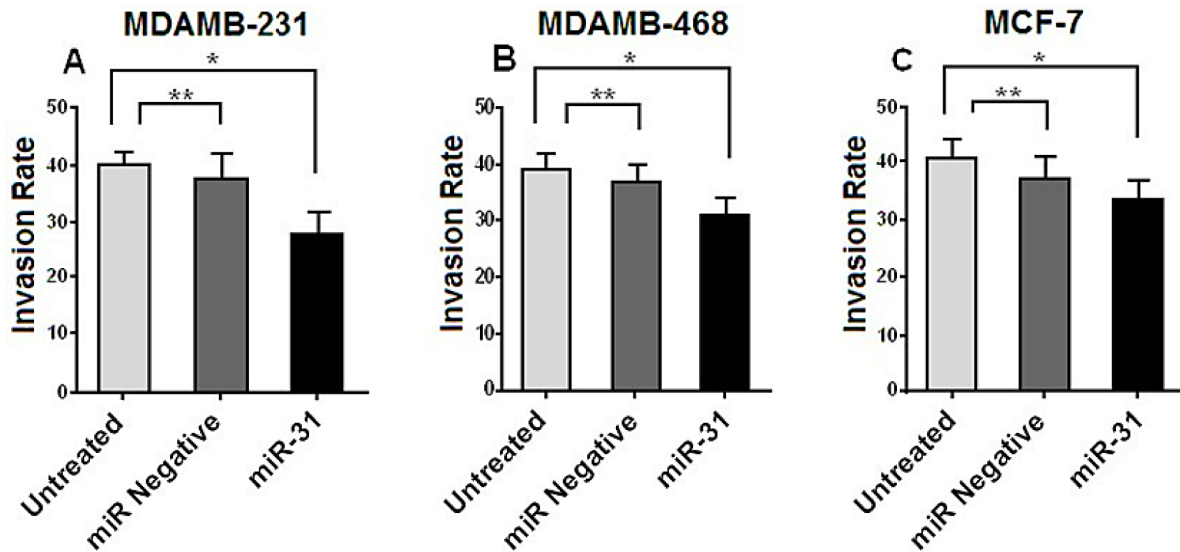


Figure 5. Cell invasion rate in untreated, treated without miR-143 and treated with miR-143 in MDAMB-231 (A), MDAMB-468 (B) and MCF-7 (C) cell lines (*in vitro*). The miR-143 was downregulated in MDAMB-231, MDAMB-468 and MCF-7 cells. (A) * $p=0.099$ and ** $p=0.018$; (B) * $p=0.699$ and ** $p=0.87$; (C) * $p=0.79$ and ** $p=0.81$.

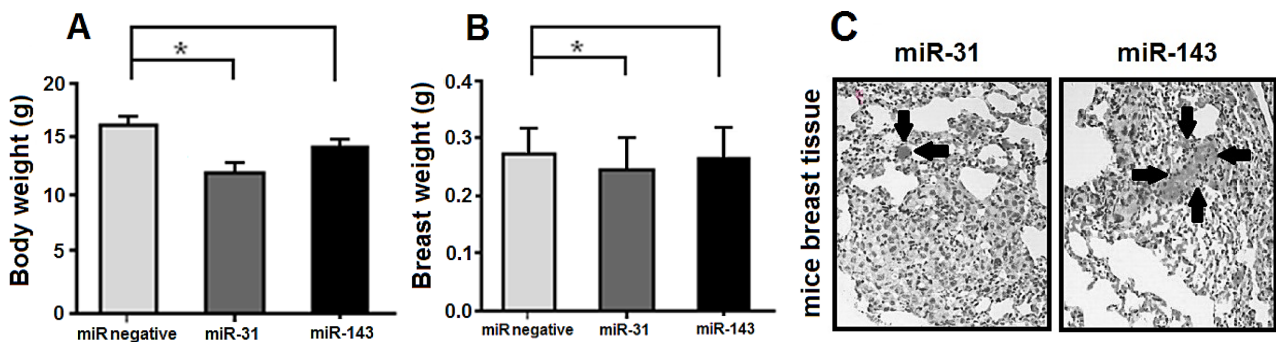


Figure 6. Five week old female BALB/c nude mice were injected with MDA-MB-231 MDAMB-468 and MCF-7 cells. The body weights (A) and the breast weights (B) were measured after six weeks. The obtained results showed that the body and breast weight in miR-31 group was significantly more than in the control group (* $p<0.05$). (C) the hematoxylin and eosin staining of mice breast tumor tissue from miR-31 and miR-143. The results showed that the number of tumor foci (arrows) in miR-143 group was more than in miR-31 group.

10 years, studies have dealt with miRs with regard to expression profiling, mechanism of action, functional characterizations and clinical implications. Furthermore, cancer biologists have identified the fundamental role of miRs in cancer progression and metastasis.

Breast cancer includes several subtypes, which result in different biological phenotypes. On the other hand, many different miRs are involved in the determination and regulation of these subtypes. Nowadays, determining the role of miRs in human body, such as regulation of expression, secretion and expression in tissues are substantially important. Previous studies [3,5] enhanced our knowledge about the miRs, and today miRs are known as vital factors in the diagnosis and treatment of breast cancer.

Genomic studies showed reduction of expression or deletion of the miR-31 genomic locus in many cancers [32]. This study has also confirmed the miRs' positive impact on the inhibition of breast cancer metastasis. Moreover, this study had demonstrated that the miR-31 expression in MDA-MB231 metastatic cell line was strongly reduced compared to MCF-7 non-metastatic cancer cell line. The miR-31 and miR-143 expression was not involved in the growth and proliferation, but they affected cell migration and invasion. This ability of miRs inhibits multiple metastatic targets and enables inhibition on the several stages of metastasis cascade.

According to the types of miRs' function and their position in the patient's tissue there are two methods of treatments with the miRs (antagonist

and mimic). Antagonists are used to miR function inhibition which has gained function, and mimic are used for miR function recycling that have lost their function [33]. According to previous studies, methods based on miR-mimic are superior compared to antagonistic methods [34,35]. Therefore, the present study was performed using mimic methods. Studies have shown that miRs, especially those that target metastatic cancers, can be bona fide tumor suppressors [32]. Thus, according to our

results, miR-31-mimic and miR-143-mimic can be ideal options to consider invasion and metastasis inhibition in breast cancer, but miR-31 is highly efficient than miR-143 in the inhibition of invasion and metastasis in breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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