

**Research Article**

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**miR-3614-3p suppresses cell aggressiveness of human breast cancer by targeting AKT3 and HDAC1 expression****Zhenzhen Wang<sup>1</sup>; Xintao Jing<sup>2</sup>; Zhenghao Zhao<sup>3</sup>; Fang Li<sup>2</sup>; Huahua Zhang<sup>4</sup>; Yanke Chen<sup>2\*</sup>; Chen Huang<sup>2\*</sup>**<sup>1</sup>Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi, China.<sup>2</sup>Department of Cell Biology and Genetics/Key Laboratory of Environment and Genes Related to Diseases, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi, China.<sup>3</sup>Medical Research and Experimental Center, Medical College, Yan'an University, Yan'an, Shaanxi, China.**\*Corresponding Authors: Yanke Chen & Chen Huang**

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**Abstract**

**Purpose:** MicroRNAs (miRNA) have been reported in the regulation of various pathobiological progression in cancer. Our recent study has reported that miR-3614-3p significantly suppressed the proliferation of Breast Cancer (BC) cells through the downregulation of its host gene TRIM25. However, the other functional role of miR-3614-3p migration and invasion in BC and its mechanism have not been investigated thoroughly.

**Materials and methods:** The MDA-MB-231 and MCF-7 BC cell lines were purchased. The cell line expression levels of miR-3614-3p and AKT3/HDAC1 were determined by quantitative real-time PCR (qPCR). The wound healing assay and transwell migration assay were determined. We next measured protein levels of AKT3/HDAC1 by Western blot. Finally, we investigated the role of AKT3/HDAC1 using siRNA; and confirmed the targeting of 3'UTR of AKT3 and HDAC1 through miR-3614-3p using a luciferase reporter assay.

**Results:** In the present research, we studied that overexpression of miR-3614-3p markedly suppressed tumor cell invasion and migration independent TRIM25, whereas through regulated another targets AKT3 and HDAC1 expression. Notably, TRIM25 is also a target gene of miR-3614 which bind to pri-miR-3614 caused TRIM25 silence.

**Conclusion:** miR-3614-3p is an anti-oncogene that can suppress breast cancer cell aggressiveness by targeting AKT3 and HDAC1, which reveals the potential values of miR-3614-3p for suppression of metastasis of BC.

**Keywords:** Breast cancer; miR-3614; AKT3; HDAC1.

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## Introduction

Breast Cancer (BC) is one of the most common type of cancer and the most prevalent aggressive malignancies in women. BC is treated with combinations of surgery, chemotherapy, radiation therapy and hormone therapy [1-3]. In spite of the advances in treatment, most of patients with BC have a poor prognosis because of the high frequency of distant metastasis or tumor recurrence. Thus, it is necessary to make deeper study on the molecular mechanisms involved in the BC process which is essential for the development of more effective therapies for BC patients.

MicroRNAs (miRNAs) are an abundant of endogenous non-coding RNAs (19-24 in length) that regulate gene expression by binding to 3'-UTR of many target mRNA resulting in RNA degradation, affecting mRNA translation and transcription [4]. Lots of evidence reveals that miRNAs play an important role in biological processes including cell invasion, proliferation, migration and apoptosis [5-8]. Numerous miRNAs are aberrantly expressed in many types of cancer, including gastric cancer, hepatocellular carcinoma, leukemia, lung and liver cancer, suggesting that they may function as either tumor oncogenes or suppressors [9-13].

MiR-3614 is an intragenic miRNA, located on chromosome 17 and its host gene is tripartite motif-containing 25 (TRIM25). In our previous study, we observed that miR-3614-3p suppresses the TRIM25 expression by binding to its 3'-UTR and forced overexpression of miR-3614-3p inhibits proliferation of BC cells through the downregulation of TRIM25 [14]. However, the other functional role of miR-3614-3p migration and invasion in BC is poorly understood. Thus, we speculate whether miR-3614 affects the invasion and migration of BC by regulating the expression of the downstream gene TRIM25.

Here, we identify miR-3614-3p inhibited the migration of MDA-MB-231 and MCF-7 cells and miR-3614-3p exerted its biological function by targeting AKT3 and HDAC1. Taken together, our results suggested that miR-3614-3p suppresses cell aggressiveness of BC by downregulating AKT3 and HDAC1 expression.

## Materials and methods

### Cell culture

The cells used in this study consisted of three cell lines MDA-MB-231, MCF-7 and 293T(HEK) cells (the Institute of Biochemistry and Cell Biology, Shanghai, China), were grown in Falcon culture dishes with DMEM medium, which supplemented with 1% antibiotics and 10% fetal bovine serum (all from Sigma) in a CO<sub>2</sub>-regulated incubator in humidified 95% air and 5% CO<sub>2</sub> atmosphere.

### Plasmid construction

The miR-3614 expression vector (pre-miR-3614) and the control vector were synthesized and cloned into the pcDNA<sup>TM</sup>6.2-GW/EmGFPmiR plasmids (Invitrogen). The binding site of miR-3614 in the 3'-UTR of human AKT3 and HDAC1 mRNA were constructed and cloned in between the Xho I and Sac I sites of the pmirGLO Dual-luciferase miRNA target expression vector (Promega). We constructed pmirGLO- AKT3/ HDAC1 Wild-Type (WT) and pmirGLO- AKT3/ HDAC1 Mutant-Type (MUT), as described previously [14] SiRNAs, anti-miR-3614 and

their respective negative control RNAs were purchased from Gima (Shanghai, China). The plasmid and miRNA sequences were as follows: pre-miR-3614: GGUUCUGUCUUGGGCCA-CUUGGAUCUGAAGGCUGCCCUUUGCUCUCUGGGGUAGC-CUUCAGAUCUUGGUGUUUUGAAUUCUUACU; inhibitor miR-3614-3p: AAAACACCAAGATCTGAAGGCTA.

### Quantitative real-time PCR (qRT-PCR).

Total cellular RNA was extracted with TRIzol Reagent (Invitrogen Carlsbad, USA). Real-time PCR was performed with the Prime Script RT Reagent Kit and the SYBR Premix Ex Taq II Kit (Takara, Japan) to detect the expression of mature miR-3614 and AKT3/HDAC1 mRNA. Samples were run using an IQ5 Multicolor qRT-PCR Detection System (Bio-Rad, Laboratories, CA, USA). Forward and reverse primers were used as follows: AKT3, GCAGAGGCAAGAAGAGGAGA and ACTTGCCTTCTCTGAACCA; HDAC1, CATCTCCTCAGCATTGGCTT and TATTATGGACAAGGC-CACCC.

### Western blot analysis

The BC cells were harvested with RIPA lysis buffer (CST, Boston, China) after 48 h of transfection. The equal amounts of protein lysates were separated by 10% SDS-PAGE and transferred onto a methanol-activated PVDF membrane (Millipore, USA). The membranes were incubated with the following primary antibodies: AKT3 (1:2000, Abcam), HDAC1 (1:1000, Abcam) overnight at 4 °C. The membranes were incubated with secondary antibody for 2 h at room temperature, followed with ECL reagent (Pierce, USA) for chemiluminescence detection, as described previously [14].

### Luciferase reporter assay

The AKT3/ HDAC1-WT or AKT3/ HDAC1-MUT vector was cotransfected with pre-miR-3614 plasmid or corresponding control pcDNA6.2-GW into HEK293T cells. The cells were lysed for luciferase activity 24 h after transfection. Firefly and Renilla luciferase were measured by the Dual-Glo Luciferase Assay System (Promega, USA).

*Wound healing assay.* The MCF-7 and MDA-MB-231 cells were plated in 6-well culture plates. The cell monolayer was scraped using a P-10 micropipette tip and the suspension cells were removed with PBS. The cells were allowed to migrate in reduced serum DMEM medium in an incubator. At 0h, 24h and 48 h, three fields of the wound area were taken with a phase-contrast microscope.

### Transwell migration assay

The migration assay was performed using Transwell chamber (Millipore, USA). The BC cells were seeded into the upper chambers with serum-free medium ( $1.0 \times 10^4$  cells), and the lower chambers contained the DMEM medium with 10% FBS. After incubation for 24 h, the cells were fixed and stained with crystal violet. Migrated MCF-7 and MDA-MB-231 cells were manually counted under an inverted light microscope.

### Statistical analysis

Results are presented as the mean  $\pm$  SEM of three independent experiments at least. Significance was established with

the SPSS 22.0 software (IBM, USA). Student's t-test and ANOVA analysis were used if the quantitative data between groups show normal distribution. If not consistent with the normal distribution, using the Wilcoxon-Mann-Whitney test. Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Overexpression of miR-3614-3p inhibits migration and invasion of BC cells

To explore the biologic significance of miR-3614-3p in BC, MCF-7 and MDA-MB-231 cells were transfected with pre-miR-3614 plasmid to overexpress miR-3614-3p. Additionally, miR-3614-3p was significantly downregulated by the inhibitor of miR-3614-3p. The efficacy of transfection was tested by qRT-PCR ( $P < 0.01$ , Figure 1A). As shown in Figure 1B, wound healing assays revealed that overexpression of miR-3614-3p required a longer time to heal in comparison with that of control cells. In addition, transwell migration assay showed that overexpression of miR-3614-3p dramatically inhibited MCF-7 and MDA-MB-231 cells migration compared with control cells (Figure 1C). In contrast, BC cells derived from the inhibitor of miR-3614-3p displayed a higher ability of migration compared with the control cells. Collectively, these results demonstrated that miR-3614-3p is capable of manipulating aggressive of BC cells.

### Effects of TRIM25 siRNA on BC cell metastasis.

In our previous study we have proved that miR-3614-3p can inhibit BC cells proliferation by targeting TRIM25. To investigate whether TRIM25 siRNA is able to influence tumor cell metastasis, cell migrated and invasive capability was used in MCF-7 and MDA-MB-231 cells. Importantly, siTRIM25-transfected cells had similar wound healed rate with scrambler-transfected cells (Figure 2A,  $P > 0.05$ ), however, compared with control groups, migration of BC cells was reduced following the transfection of TRIM25 siRNA (Figure 2B,  $P < 0.05$ ). These data suggested that TRIM25 had no big effect on the metastasis of BC cells, there might be other target genes to influence BC cell metastasis.

### The mechanism of miRNA-3614-3p self-silence

In addition, we hypothesized that the mature miR-3614-3p gets cleaved by the Dicer complex and binds to pri-miR-3614. We assumed that through this mechanism, miR-3614-3p would silence itself. In order to prove this hypothesis, we cloned pri-miR-3614 fragments into the pmiRGLO vector and generated mutations in them (Figure 3A). As expected, the relative luciferase activity was significantly decreased when pre-miR-3614 and pri-miR-3614-pmiRGLO vector were co-transfected into HEK293T cells, compared with cells transfected with only pmiRGLO vector. However, the mutation in pri-miR-3614 did not affect relative luciferase activity. Conversely, transfection of the miR-3614-3p inhibitor enhanced the luciferase activity (Figure 3B). These results together suggest that miR-3614-3p can silence itself.

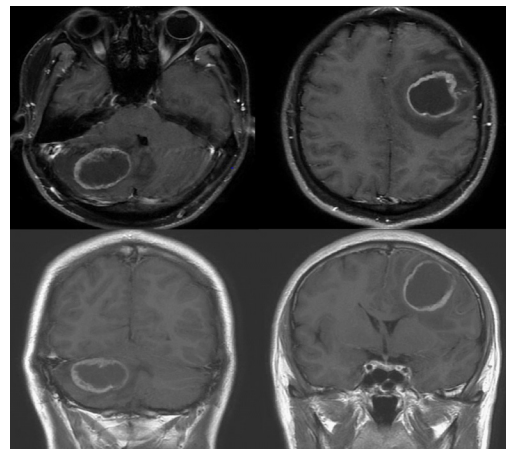
### AKT3 and HDAC1 are Direct Targets of miR-3614-3p

To elucidate the molecular mechanism by which miR-3614-3p exerts its inhibitory effect on BC cells, we searched for miR-3614-3p target genes using prediction programs and found that well-matched miR-3614-3p binding sites in the AKT3 and HDAC1 3' UTR (Figure 4A). To validate the hypothesis that AKT3 and HDAC1 are targets of miR-3614-3p, a dual-luciferase re-

porter system containing AKT3 and HDAC1 3' UTR-WT/MUT was used. miR-3614-3p and a reporter plasmid or pmiRGLO control vector were co-transfected in HEK293T cells. As illustrated in Figure 4B, miR-3614-3p clearly reduced the firefly luciferase activity of AKT3 and HDAC1-WT at 24 h compared to the MUT-type and control. To confirm whether the loss of miR-3614-3p is an important factor of BC malignancy by upregulating AKT3 and HDAC1 expression, we performed that qRT-PCR and Western blot analysis and found that overexpression of miR-3614-3p dramatically decreased the mRNA and protein level of AKT3 and HDAC1 (Figure 4C). Moreover, we found that the AKT3 and HDAC1 levels were overexpressed in BC tissues than compared to normal tissue controls from TCGA database ( $P < 0.0001$ ). Likewise, the same results AKT3 and HDAC1 mRNA and protein overexpressed in MCF-7 and MDA-MB-231 cell lines, in which miR-3614-3p was downregulated (Figure 4D). Taken together, the above data indicate that miR-3614-3p directly regulates AKT3 and HDAC1 expression in MCF-7 and MDA-MB-231 cells.

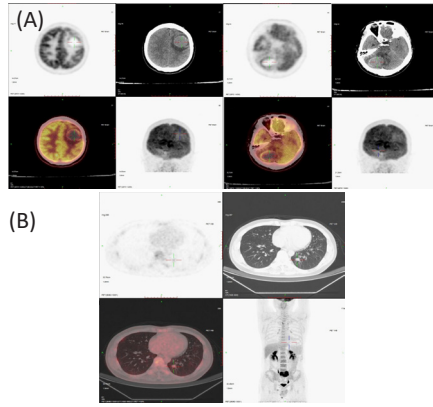
### Silencing of AKT3 and HDAC1 reduced BC cells invasiveness

To further validate that miR-3614-3p suppressed BC cell migration by regulating AKT3 and HDAC1, we inhibited AKT3 and HDAC1 mRNA and protein expression by their knockdown siRNA (Figure 5A). Moreover, silencing of AKT3 and HDAC1 suppressed cell wound healing and migration ability (Figure 5B&5C), which followed the same trend as miR-3614-3p in MCF-7 and MDA-MB-231 cells. Based on these findings, AKT3 and HDAC1 are essential and functional downstream mediators of miR-3614-3p in BC.

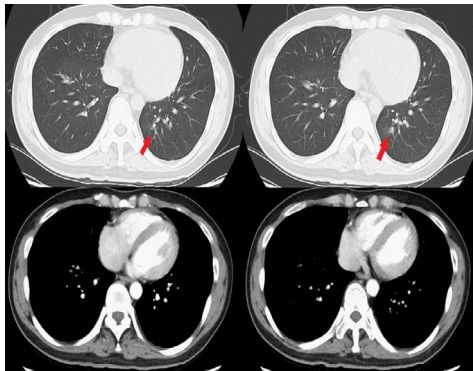


**Figure 1:** Ectopic expression of miR-3614-3p ameliorates BC migration and invasion in vitro. (A) miR-3614-3p was assessed in MCF-7 and MDA-MB-231 cells after transfection with pre-miR-3614 expression vector and anti-miR-3614. (B) Wound-healing assays showed that miR-3614-3p depressed cell migration, images were captured at 0, 24 and 48 hours after scratching. (C) Transwell assays showed that miR-3614-3p depressed cell metastasis (upper panel: migration assays; lower panel: invasion assays). Experiments were repeated at least 6 times with similar results, and error bars represent  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

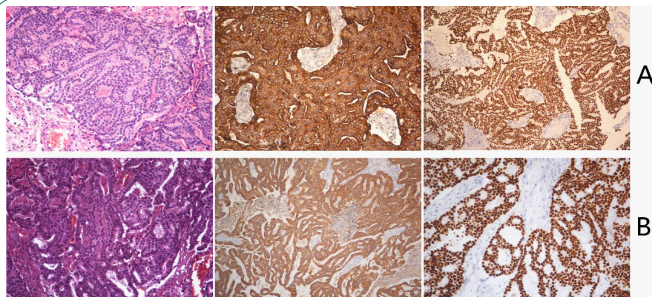




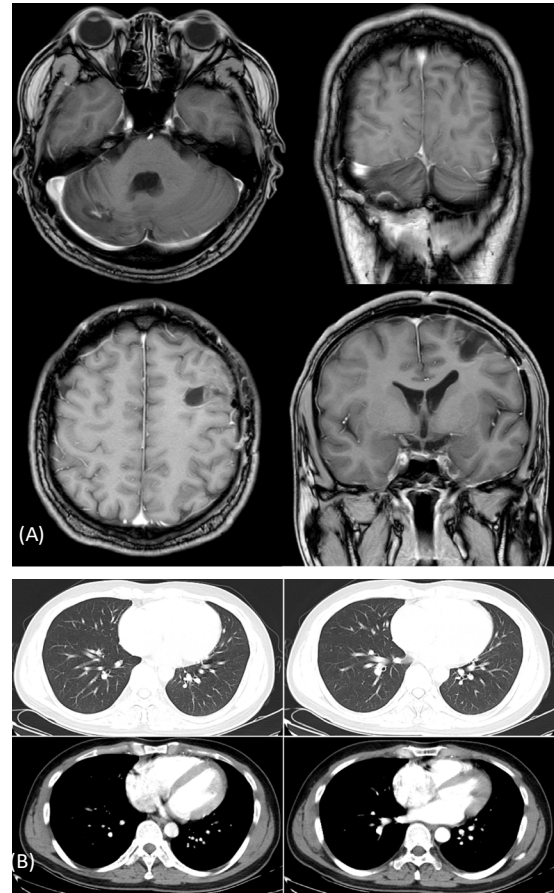
**Figure 2:** The effect of TRIM25 knockdown siRNA on BC cell metastasis. **(A)** Wound-healing assays showed that siTRIM25 had no obvious difference as compared with scrambled. **(B)** Transwell assays showed that siTRIM25 inhibited cell metastasis (upper panel: migration assays; low panel: invasion assays). Experiments were repeated at least 6 times with similar results, and error bars represent  $\pm$  SD. \*\*\* $p < 0.001$



**Figure 4:** AKT3 and HDAC1 are experimentally validated as direct targets of miR-3614-3p in human BC cells. **(A)** The binding sites of miR-3614-3p at the 3'-UTR of AKT3 and HDAC1 were predicted by bioinformatics. **(B)** Luciferase reporter vectors (including wild or mutant type AKT3/HDAC1-3'-UTR) were cotransfected in combination with miR-3614 or Control into HEK293T cells. Luciferase activity was examined by a dual-luciferase assay. **(C)** AKT3/HDAC1 mRNA and protein expressions were assessed in MCF-7 and MDA-MB-231 cells after transfection of pre-miR-3614 expression vector/anti-miR-3614. **(D)** The expression data RNA-Seq analysis of AKT3 and HDAC1 expression in BC tissues ( $n = 143$ ) and normal tissues ( $n = 502$ ). RNA-Seq analysis used data download from TCGA. AKT3/HDAC1 mRNA and protein expressions were assessed in BC cell lines. Data are shown as mean  $\pm$  SEM (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ )



**Figure 3:** The mechanism of pre-miR-3614 self-silence. **(A)** miR-3614-3p and miR-3614-5p are highly conserved across species and they have binding sites within the pre-miR-3614. **(B)** The luciferase assay was performed in HEK293T cells in which miR-3614 or anti-miR-3614-3p was co-transfected with or without pGLO-pre-miR-3614 wild-type or pGLO-pre-miR-3614 mutant vector. Data are shown as mean  $\pm$  SEM (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 5:** AKT3 and HDAC1 are the functional mediators downstream of miR-3614-3p in BC cells. **(A)** qRT-PCR and western blot were performed to examine the expression of AKT3 and HDAC1 after transfection with AKT3/HDAC1 siRNA. **(B)** Wound-healing assays showed that si-AKT3/HDAC1 depressed cell migration, images were captured at 0, 24 and 48 hours after scratching. **(C)** Transwell assays showed that si-AKT3/HDAC1 depressed cell metastasis (upper panel: Migration assays; low panel: invasion assays). Experiments were repeated at least 6 times with similar results, and error bars represent  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Discussion

In our previously study, we showed that miR-3614-3p was downregulated in breast cancer tumors in comparison with the normal tissues. Moreover, miR-3614-3p was reduced in MCF-7 and MDA-MB-231 cell lines compared with a normal cell line HBL-100. In current study indicated that miR-3614-3p could reduce cancer cell invasion and migration, but the inhibition of the invasion and migration is more significant in invasive breast cancer cells (MDA-MB-231) than noninvasive breast cancer cells (MCF-7) using transwell matrigel and wound healing assay. In contrast, anti-miR-3614-3p oligonucleotides were used for loss of function studies to silence miR-3614-3p expression. The inhibitory effect revealed a significant contribution to the tumorigenicity of MCF-7 and MDA-MB-231 cells. Therefore, these data suggested that miR-3614-3p functioned as a tumor suppressor for the cell aggressiveness in BC. To this end, validation by in vivo assays is needed for further research.

It is necessary to identify the downstream functional targets to elucidate its biological effects. In our previously study, we have reported that overexpression of miR-3614-3p dramatically inhibits breast cancer cell growth through the downregulation of TRIM25. However, in this study we found that TRIM25 had no big effect on the metastasis of BC cells and there might be other

target genes to influence BC cell metastasis. Through online prediction using TargetScan, AKT3 and HDAC1 were screened out to be the target genes of miR-3614-3p. AKT, also named as PKB, is important in maintaining normal cellular functions. Previous studies have already confirmed the correlation between tumor development and AKT3 [15-18]. Yong-Xia Wang et al [19]. verified that miR-384 could significantly suppress the proliferation of colorectal cancer by directing targeting AKT3, and microRNA-433 targets AKT3 and inhibits cell proliferation and viability in breast cancer [20]. Rui Li and YING MA showed that miR-29a and miR-144 suppresses growth and metastasis in papillary thyroid carcinoma and hepatocellular carcinoma by targeting AKT3 [21,22].

Histone Deacetylases (HDACs) are posttranslational modifiers that deacetylate proteins [23]. Numerous investigations have demonstrated that HDAC1 involved in the tumor progression [24-27]. For example, miR-34a regulates HDAC1 expression to affect the proliferation and apoptosis of hepatocellular carcinoma and miR-761 inhibits colorectal cancer cell proliferation and invasion through targeting HDAC1 [28]. Junyi He revealed that HDAC1 promoted migration and invasion binding with TCF12 by promoting EMT progress in gallbladder cancer. Moreover, Liu X et al. found that Upregulation of HDAC1 is a crucial event in the development of drug resistance to current treatments in ovarian cancer. Thus, targeting HDAC1 by enhancing c-Myc-dependent miR-34a expression might be an effective strategy for increasing the efficacy of cisplatin treatment [29,30].

Our study identified that AKT3 and HDAC1 overexpressed in breast cancer tissues and cell lines. Furthermore, AKT3 and HDAC1 were direct downstream targets of miR-3614-3p and upregulation of miR-3614-3p significantly reduced the expression of AKT3/HDAC1 and decreased the luciferase reporter activity of AKT3/HDAC1 WT 3'-UTR but not MUT 3'-UTR. Besides, we showed that silencing of AKT3 and HDAC1 suppressed cell wound healing and migration ability. Importantly, in our previous study have provided that TRIM25 is not only a host gene, but also a target gene of miR-3614-3p in BC. In this result, we found that miR-3614-3p significantly binding to pri-miR-3614 at the 3'-UTR of TRIM25, suggesting that miR-3614-3p could silence itself. Taken together, these data provide competent evidences to support that miR-3614-3p exerts its suppressive effect on BC, at least partly, through inhibiting AKT3 and HDAC1 expression.

## Conclusions

In summary, our results revealed that miR-3614-3p was downregulated together with AKT3 and HDAC1 overexpression in BC tissues and cell lines. Moreover, miR-3614-3p could regulate the migration and invasion of MCF-7 and MDA-MB-231 cells by targeting AKT3 and HDAC1 expression. Thus, we consider that miR-3614-3p might be a biomarker in target therapy of breast cancer especially the invasive form of this disease.

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