

## Original Article

# miR-148a overexpression inhibits cell proliferation and induces cell apoptosis by suppressing the Wnt/ $\beta$ -catenin signal pathway in breast cancer MCF-7 cells

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**Abstract:** Increasing evidence shows that pathogenesis for breast cancer remains complicate and recent study refers that the down-regulated miR-148a is associated with the diagnosis for breast cancer. This study was aimed to investigate the molecular correlation between miR-148a and Wnt signal pathway in breast cancer development. The expression of miR-148a in normal breast Hs578Bst cells and MCF-7 cells were detected using the RT-PCR. Cell viability and apoptosis were analyzed using the MTT assay and Annexin V-FITC assay respectively. Furthermore, the Wnt/ $\beta$ -catenin signal pathway-related protein expression was measured using the western blotting analysis. Compared to the normal Hs578Bst cells, miR-148a expression was decreased in MCF-7 cells. The MCF-7 cell viability was significantly decreased while the percentage of apoptosis was promoted by the overexpressed miR-148a. Also, the mRNA and protein levels for Wnt1,  $\beta$ -catenin, and C-myc were decreased by the overexpressed miR-148a. Taken together, our study revealed that the overexpressed miR-148a might function as a suppressor in the development of breast cancer by suppressing the activation of Wnt/ $\beta$ -catenin signal pathway.

**Keywords:** Breast cancer, miR-148a, Wnt/ $\beta$ -catenin signal pathway, cell proliferation, cell apoptosis

## Introduction

Breast cancer remains to be one of the most common malignancies among females globally, which has a high morbidity and with -465,000 mortalities from breast cancer annually worldwide [1]. In recent decades, various studies have devoted efforts to the pathogenesis and treatment methods exploration for breast cancer [2-4]. However, the cure methods for breast cancer including either drug or surgery still remain insufficient due to its complicate pathogen mechanism [5, 6]. Hence, it is necessary to explore several novel and a useful therapeutic target for breast cancer treatment.

Previous evidences show that early diagnosis reduces the rate of mortality from breast cancer [7-9]. microRNAs (miRNAs) are some endogenous, highly conserved non-coding RNAs 20- to 22-nt in length that function in a variety

kinds of biological processes at the transcriptional or post-transcriptional level via targeting the 3'-UTR of genes [10]. Increasing evidence refers that various miRNAs are involved in the development or biological processes for breast cancer, such as miR-10b, miR-21, and miR-125b [11-13]. Also, miR-148a down-regulation has been reported to be involved in many kinds of cancers including urothelial carcinoma, gastric cancer, and breast cancer through intricate mechanisms [14, 15]. Luo et al report that miR-148a is down-regulated in breast cancer and acts as a specific tool for diagnosis of breast cancer [16].

Wnt is a proto-oncogene that isolated from the mammary gland of mice, functioning as a regulator in cell proliferation and apoptosis under the regulation of many factors in cells [17, 18]. The Wnt signal pathway is consist of Wnt, Frizzled, E-cadherin,  $\beta$ -catenin, Dishevelled and

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**Table 1.** Primers used for targets amplification in this study

Target	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
Wnt1	Sense	TACCTCCAGTCACACTCCCC
	Antisense	CCATGGCAGGAGAATAGGAA
$\beta$ -catenin	Sense	GGGCGGCACCTTCTACTTTC
	Antisense	AGCTCCCTCGCGGTTTCAT
C-myc	Sense	CTACCAGCAGCAGCAGAGC
	Antisense	CGTCCGGGTCGCAGATGAA
miR-148a	Sense	AACTCCAGCTGGGTCAGTGCACACAGAA
	Antisense	CTCAACTGGTTCGGAGTCGGCAAITCAGTRGAGACAAAGTT
U6	Sense	CGCTTCACGAATTTGCGTGTTCAT
	Antisense	AACGCTTCACGAATTTGCGT

other proteins [19]. The combination between the nomadic  $\beta$ -catenin and Tcf/Lef caused by the dysfunction of  $\beta$ -catenin degradation, which results in the activation of the downstream proteins including cyclin D1 and C-myc, has been considered as the pivotal step for Wnt signal pathway in tumorigenesis [20]. Studies have reported that the dynamic expression of Wnt family protein regulates the cell proliferation and differentiation in breast mammary gland development [21, 22]. Recently, several studies report that some miRNAs can directly regulate Wnt signal pathway and function in breast cancer, including miR-148a [23]. However, the molecular mechanism still remains incomplete.

In the current study, we analyzed the expression of miR-148a in breast cancer MCF-7 cells and further investigated the influence of miR-148a expression on breast cancer cell proliferation and apoptosis. Besides, the effects of miR-148a expression on the Wnt/ $\beta$ -catenin signal pathway-related protein expression were analyzed. This study was aimed to investigate the correlation between miR-148a and Wnt/ $\beta$ -catenin signal pathway in breast cancer and to reveal its action. Our study may provide basis for the possible application of miR-148a in breast cancer therapeutic treatment.

### Materials and methods

#### Cell lines and transfection

Human normal breast Hs578Bst cells and human breast cancer MCF-7 cells (obtained from American Type Culture Collection) were cultured in RPMI 1640 medium (Sigma, USA) containing 10% fetal bovine serum (FBS; Sigma

in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

The overexpressed vector for miR-148a (Sangon Biotech, Shanghai, China) was transferred into the breast MCF-7 cells using the Lipofectamine 2000 protocol (Life Technologies, USA). Cells transfected with the scramble miR-148a was used as the controls.

#### Cell proliferation assay

Cell proliferation ability was assessed using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay as previously described [24]. Briefly, after transfection for 24 h, cells adjusted to 5×10<sup>3</sup> cells were used for the injection onto the 96-well plates. After 24 h, cells were centrifuged at 12,000 rpm, and then supernatant was removed. Then 20  $\mu$ L MTT was added into the cells and then cultured for another 4 h. Finally, 150  $\mu$ L dimethylsulfoxide (DMSO) was mixed with cells for 10 min to stop the reactions. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

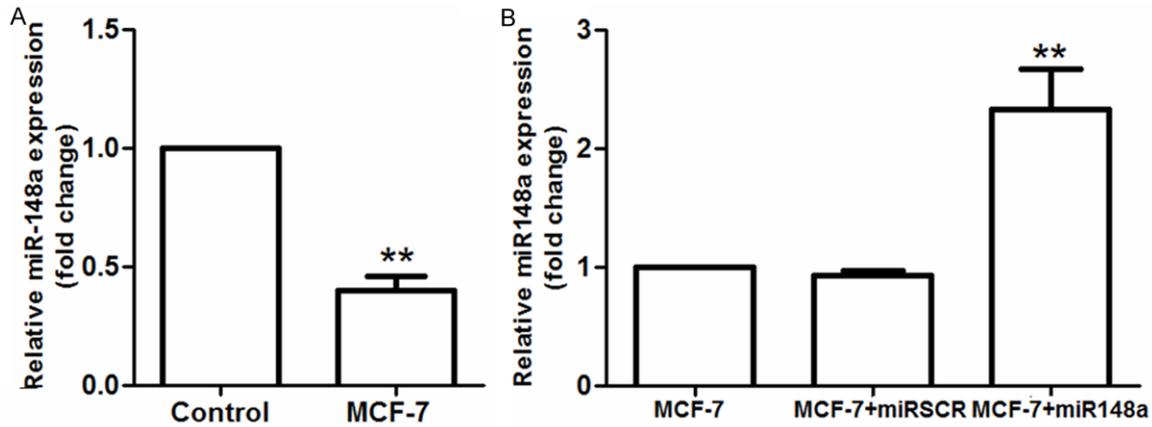
#### Cell apoptosis assay

The apoptosis cells were measured using the flow cytometry with the Annexin V-FITC cell apoptosis kit (Invitrogen, USA) according to the manufacturer's protocol. Briefly, after transfection for 36 h, cells were cultured in the fresh serum-free RPMI 1640 medium. Then total cells were harvested and washed 3 times with the PBS buffer, followed with the resuspended in the staining buffer. After that, 5  $\mu$ L of annexin-V-FITC and 5  $\mu$ L of propidium iodide (PI) were added into the cells at room temperature for 10 min. Mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

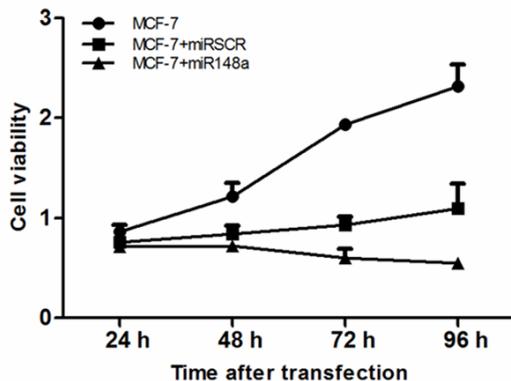
#### Real time (RT)-PCR

Total RNA extraction from the cells was performed using the TRIzol Reagent (Invitrogen) according to manufacturer's protocol. The extracted RNA was treated with RNase-free Dnase I (Promega Biotech, USA) to remove the

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**Figure 1.** Expression of miR-148a in breast cancer MCF-7 cells. A: miR-148a mRNA level in MCF-7 cells was lower than that in the control. \*\*:  $P < 0.01$ , compared to the control (normal breast Hs578Bst cell); B: The relative miR-148a level is significantly increased by the overexpressed miR-148 vector transfection in MCF-7 cells. \*\*:  $P < 0.01$ , compared to the control cells (MCF-7 cell without transfection).



**Figure 2.** miR-148a expression was correlated to the breast cancer MCF-7 cell viability. Compared to the control, the overexpressed miR-148a decreased the cell viability with time increasing after transfection.

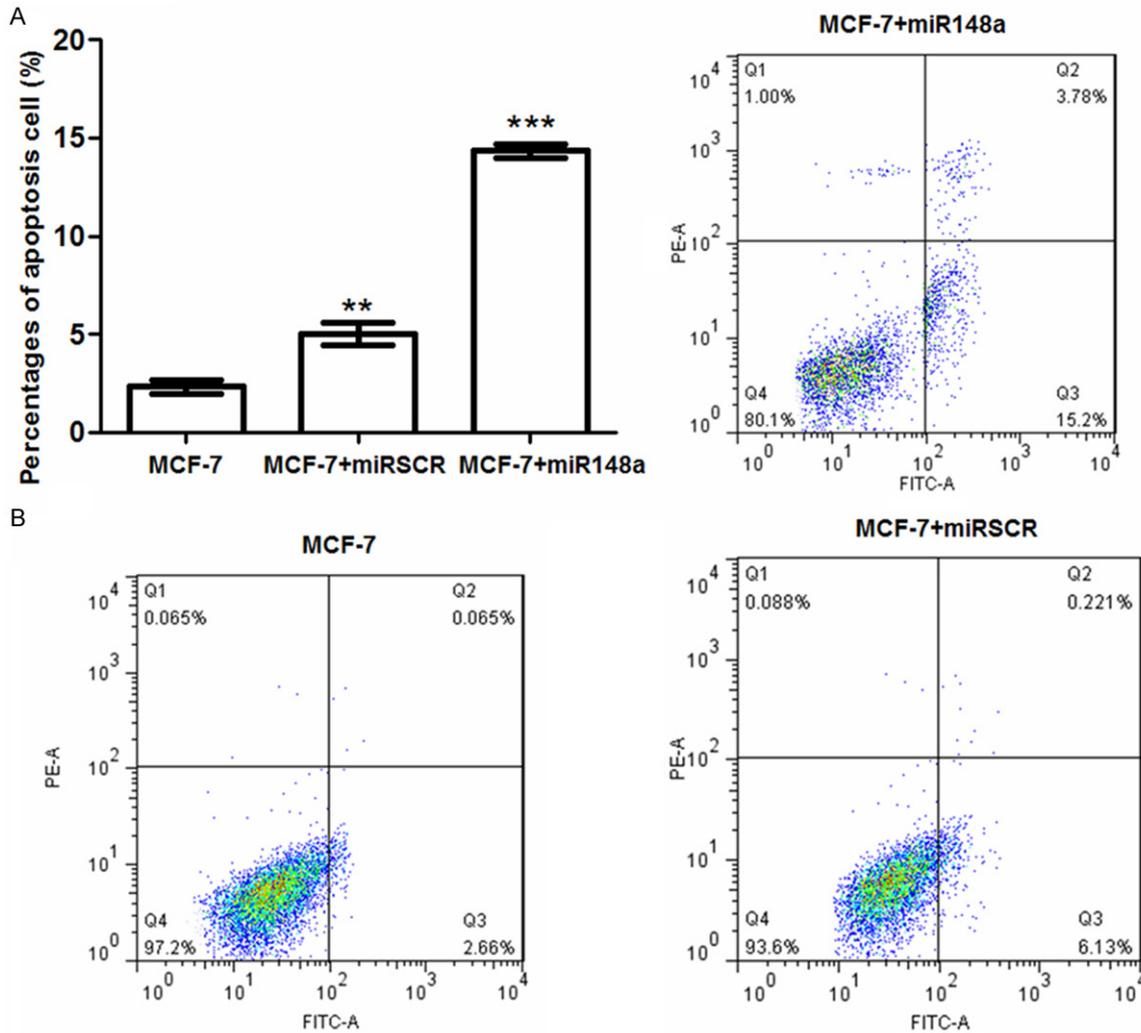
mixed DNA, and concentration and purity were measured using SMA 400 UV-VIS (Merinton, Shanghai, China). The purified RNA ( $0.5 \mu\text{g}/\mu\text{L}$ ) dissolved in nuclease-free water was used for cDNA synthesis with the PrimerScript 1<sup>st</sup> Strand cDNA Synthesis Kit (Invitrogen). Complementary DNA (cDNA) was produced using reverse transcriptase (iScript<sup>TM</sup> cDNA Synthesis Kit; Bio-Rad Laboratories). Expressions of targets were performed in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control for target gene expression, U6 was used as the internal control for the miRNA expression. Primers used for targets amplification were shown in **Table 1**.

### Western blotting

Cells collected at 48 h after transfection were lysed with radioimmunoprecipitation assay (RIPA; Sangon Biotech) lysate containing phenylmethanesulfonyl fluoride (PMSF; Sigma), and then were centrifuged at 12,000 rpm at  $4^\circ\text{C}$  for 10 min. Concentration for protein was detected using BCA protein assay kit (Pierce, Rochford, IL). For western blotting,  $50 \mu\text{g}$  protein per cell lysate was subjected onto a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto a polyvinylidene difluoride (PVDF) membrane (Mipprore). The PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk at room temperature for 1 h. Then the membranes were incubated with rabbit anti-human antibodies (Wnt 1,  $\beta$ -catenin, and C-myc; 1:100 dilution, Invitrogen) and overnight at  $4^\circ\text{C}$ . Then membrane was incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with  $1\times$  TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

### Statistical analysis

All experiments were conducted independently for 3 times. Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis



**Figure 3.** Effects of miR-148a expression on breast cancer MCF-7 cell apoptosis. A: The percentage of apoptotic cells was significantly increased by the overexpressed miR-148a compared to the control; B: Annexin V-FITC assay showed that the percentage for apoptotic cells in miR-148a overexpressed group was about 15.2%, which is higher than that in the control group (2.66%). \*:  $P < 0.01$ ; compared to the control (MCF-7 cell without transfection).

for data was calculated using the graph prism 5.0 software (GraphPad Prism; San Diego, CA). Independent sample t-test was used to calculate the difference between two groups. The  $P < 0.05$  was considered as statistically significant.

## Results

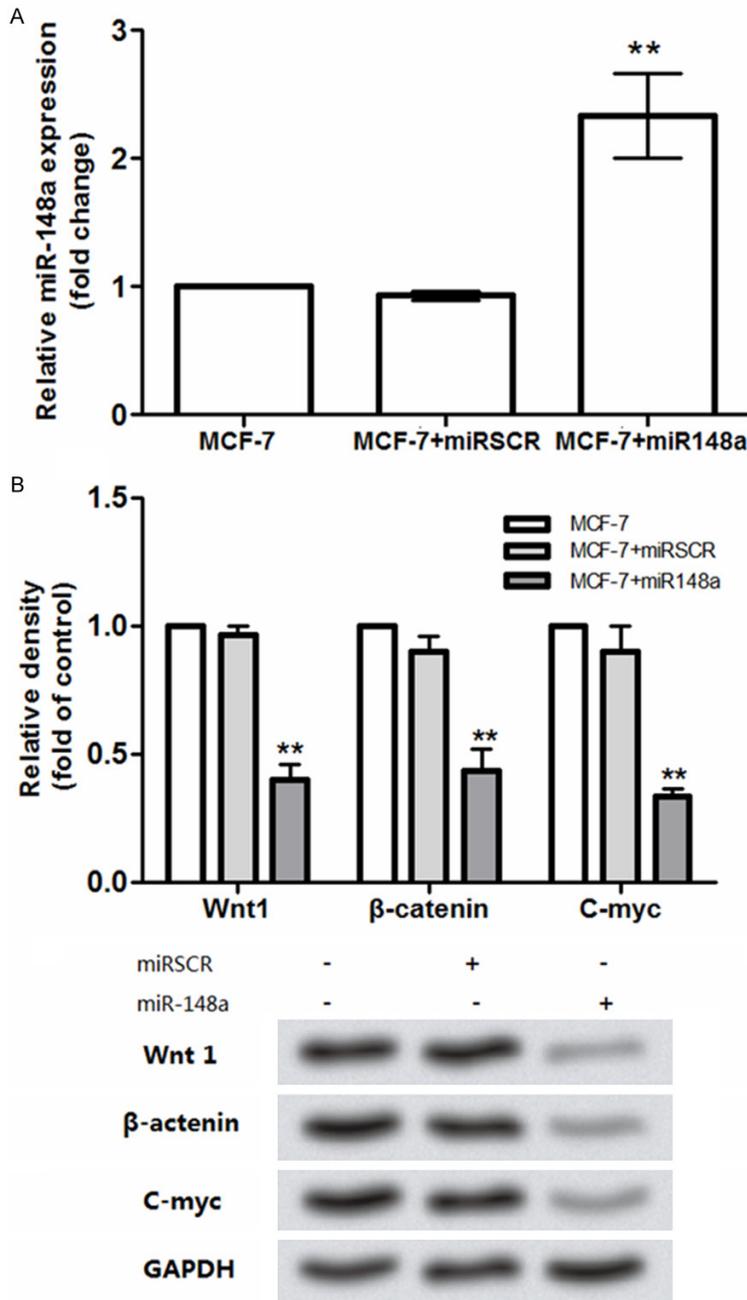
### Expression of miR-148a in breast cancer MCF-7 cells

The expression of miR-148a in MCF-7 cells was detected using the RT-PCR (Figure 1). The results showed that miR-148a was significantly down-regulated in MCF-7 cells compared to

the normal control cells ( $P < 0.01$ ; Figure 1A). However, its expression was significantly increased when cells were transfected with the overexpressed miR-148a vector ( $P < 0.01$ ; Figure 1B).

### miR-148a overexpression decreased the MCF-7 cell viability

Compared to the control, cell viability was significantly decreased by the overexpressed miR-148a (Figure 2). No significant difference for the cell viability was observed between the control group and the cells transfected with scramble miRNA.



**Figure 4.** Influence of miR-148a expression on the Wnt/β-catenin signal pathway-related protein expression. A: After being transfected with the miR-148a vector, expression of miR-148a was significantly increased; B: The mRNA and protein levels for Wnt1, β-catenin, and C-myc were significantly decreased by the overexpressed miR-148a in MCF-7 cells. \*\*: P<0.01, compared to the control (MCF-7 cell without transfection).

*miR-148a overexpression induced MCF-7 cell apoptosis*

We further analyzed the effects of miR-148a expression on breast cancer cell apoptosis (Figure 3). The results showed that the apop-

otic cells were significantly increased by the overexpressed miR-148a (about 6.13%) compared to the control (about 2.66%) (P<0.001; Figure 3A and 3B), indicating that miR-148a expression was positively correlated to breast cancer cell apoptosis.

*Effects of miR-148a expression on the Wnt/β-catenin signal pathway-related protein expression*

Since the results revealed that miR-148a expression was correlated to the breast cancer biological processes including cell viability and apoptosis, we therefore analyzed the possible mechanism of its action (Figure 4). The results showed that the mRNA and protein levels for Wnt1, β-catenin, and C-myc were significantly decreased by the overexpressed miR-148a compared to the control (P<0.01; Figure 4B). However, no significant difference for the three proteins expression was found between the control and the scramble miRNA group (Figure 4B).

**Discussion**

Due to the complicate molecular mechanism, increasing evidence has focused on the pathogenesis research for breast cancer in recent decades, including the differentially expressed genes, miRNAs and signal pathways [11, 25]. A recent evidence showed that miR-148a is down-regulated in serum from

patients with breast cancer and acts as a diagnostic marker for breast cancer [16]. This study was aimed to investigate the possible role of miR-148a in breast cancer development and to reveal its potential mechanism. In agreement with previous data [16, 26], our results showed

that miR-148a expression was lower than that in normal breast Hs578Bst cells (**Figure 1**), indicating the correlation between miR-148a expression and the pathogen of breast cancer.

Accordingly, we investigated the effects of miR-148a expression on breast cell viability and apoptosis using the MCF-7 cells. Coincidence with the results presented in **Figure 1**, our results showed that MCF-7 cell viability was suppressed by the overexpressed miR-148a (**Figure 2**). Taylor et al said that miR-148a was used to establish a cancer-specific signature for ovarian cancer [27]. On the other hand, the results revealed that MCF-7 cell apoptosis was induced by the overexpressed miR-148a (**Figure 3**). Apoptosis plays a vital role in maintaining tissue homeostasis. Effects of miR-148a expression on breast cancer cell apoptosis has been not been widely reported. However, studies have referred that the down-regulated miR-148a promotes colorectal cancer cell apoptosis by targeting Bcl-2 [28], similar results were also found in hepatocellular cancer and gastric cancer [29]. Hence, we speculated that miR-148a abnormal expression was correlated to breast cancer pathogen via involving in the cell viability and apoptosis processes.

Dysfunction of the cell apoptosis-related signal pathway has been widely considered as the pivotal step in tumorigenesis [30]. Previous studies revealed the pivotal roles of Wnt signal pathway in the tumorigenesis and the other biological processes of cancers, including breast cancer [31]. Wissmann et al proved that WIF1, a component of Wnt signal pathway, was down-regulated in breast cancer [32]. Meanwhile, the activated Wnt1 and C-myc lead to the formation and growth of breast tumors [33, 34]. In this study, the results showed that the Wnt1 and C-myc levels were suppressed by the overexpressed miR-148a (**Figure 4**). On the other side, the role of  $\beta$ -catenin in breast cancer remains controversial. Study said that the loss of  $\beta$ -catenin resulted in the metastasis and invasion of breast cancer [35], whereas Lin et al said that the activation of  $\beta$ -catenin promoted the development of breast cancer [36]. Our results revealed that  $\beta$ -catenin was highly expressed in breast MCF-7 cells but was suppressed by the overexpressed miR-148a. Taken together, we speculated that the overexpressed miR-148a may suppress the breast cancer

development via suppressing the activation of Wnt/ $\beta$ -catenin signal pathway.

In conclusion, the data presented in our study reveals that the overexpressed miR-148a functions as a tumor suppressor for the development of breast cancer via involving in the cell proliferation and apoptosis processes and inhibiting the activation of Wnt/ $\beta$ -catenin signal pathway. This study may provide theoretical basis for the therapeutic target diagnosis of miR-148a in breast cancer. Further experimental studies are still needed to investigate the deep molecular mechanism.

### Disclosure of conflict of interest

None.

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