**Original Article**

**MicroRNA372 promotes breast cancer cell growth and metastasis by targeting LATS2**

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**Abstract:** Objective: Several microRNAs (miRNAs) have been shown to play important roles in the generation and development of breast cancer (BC) tumors. miRNA-372 (miR-372), has been shown to be indispensable for various human malignancies. However, the role of miR-372 in BC is unknown. Methods: The expression of miR-372 was assessed in two BC cell lines, BT549 and SKBr3, and in BC tissue samples by real-time reverse transcription polymerase chain reaction (qRT-PCR). Then, the effects of miR-372 on cell proliferation, the cell cycle, apoptosis, and invasion were determined using human BC cells. A luciferase reporter assay was performed to confirm the target of miR-372. Western blotting was used to determine the protein changes that occur in response to miR-372. Then, the in vivo role of miR-372 was explored using an SKBr3 tumor xenograft model. Results: Upregulation of miR-372 was observed in the tested BC cell lines and tissues. Conversely, downregulation of miR-372 not only remarkably suppressed cell proliferation and invasion but also triggered G1/S cell cycle arrest. Mouse xenograft models showed that miR-372 knockdown inhibited BC tumor growth. Moreover, we found that miR-372 regulated the expression of large tumor suppressor kinase 2 (LATS2) by directly targeting its 3’-UTR. We also observed a negative correlation between miR-372 expression and LATS2 expression in BC specimens. Silencing of LATS2 was able to restore the suppressive effect of a miR-372 inhibitor. Finally, knockdown of miR-372 suppressed the phosphorylation of proteins in the PI3K/Akt pathway. Conclusions: These findings indicate that the oncogenic effect of miR-372 in BC is mediated by targeting LATS2.

**Keywords:** MiR-372, proliferation, metastasis, LATS2, breast cancer

**Introduction**

Breast cancer (BC) is the second leading cause of cancer-related death in women, and its incidence is rising worldwide [1]. The currently available surgical and therapeutic interventions for BC are limited, the existing drugs are somewhat toxic, and they can cause serious adverse effects in some patients [2]. In addition, the long-term prognosis of BC is unsatisfactory. Therefore, elucidation of the molecular mechanisms underlying BC tumorigenesis should be useful for the development of novel treatments.

MicroRNAs (miRNAs) play important roles in various cellular functions, including cell invasion, proliferation, migration, and apoptosis [3]. In addition, it has been shown that some miRNAs function as oncogenic factors or tumor suppressors in various cancers, such as BC and lung cancer [4]. Interestingly, Wu Gang and colleagues [5] showed that miR-372 influenced the proliferation and metastasis of hepatocellular carcinoma (HCC) by downregulating the ATAD2 oncogene. However, the role of miR-372 in BC is currently unknown.

The Hippo pathway is a conserved signaling cascade consisting of the tumor suppressor kinases LATS1, LATS2, MST1, and MST2, and the adaptor proteins MOB1, MOB2, and SAV1 as core components [6]. LATS2 is the second homolog of the LATS tumor suppressor family in mammals, and the LATS2 gene is located on chromosome 13q11-12 [7]. Hideki Murakami et al. [8] reported that LATS2 suppressed the growth of malignant mesothelioma. In addition, LATS2 was shown to be involved in cell cycle modulation and suppression of the G1/S transition [9]. However, the role of LATS2 in BC is unknown.
In this study, we aimed to investigate whether miR-372 could regulate BC proliferation and metastasis. The target of miR-372 and the role of LATS2 in BC were also explored.

**Material and methods**

*Human tissues and cell lines*

For the study, 120 pairs of human BC and non-tumor tissues were obtained from patients who underwent surgery for BC at the Second Affiliated Hospital of Soochow University, and the tissue samples were preserved in liquid nitrogen. The human BC cell lines BT549 and SKBr3, and the normal mammary epithelial cell line MCF12A were purchased from American Type Culture Collection (ATCC, VA, USA). Informed consent was obtained from all patients, and the study design was approved by the ethics committee of the Second Affiliated Hospital of Soochow University. Cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% FBS at 37°C and 5% CO₂.

*Transfection*

PHLPP2 siRNA was obtained from Dharmacon (Lafayette, CO). Hsa-miR-372 inhibitor and the negative control oligonucleotides were obtained from RiboBio (Guangdong, P.R.C.). Cells were transfected with PHLPP2 siRNA (negative control) or miR-372 inhibitor RNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were used in experiments 48 hours after transfection.

*Real-time reverse transcription-polymerase chain reaction*

Total RNA was harvested with TRIzol reagent (Life Technologies, Pleasanton, CA). Then, the expression of miR-372 was evaluated by using TaqMan gene expression assays on an Agilent Mx3005P qPCR System (Santa Clara, CA). The primers used for miR-372 were as follows: forward, 5’-ACACTCCAGCTGGGAAAGTGCTGCGACTTT-3’, and reverse, 5’-GTGCAGGGTCCGAGGT-3’. The primers used for U6 were as follows: forward, 5’-CTCGCTTCGGCAGCACATATAGTT-3’, and reverse, 5’-ACGCTTCACGAATTGTGC-3’. The concentration of miR-372 was normalized to that of U6, which was used as an internal reference.

*Cell proliferation assay*

The CCK-8 assay was used to measure cell proliferation. Briefly, cells were seeded in 96-well plates. Then, 10 µL of CCK-8 was added to each well, and the plate was incubated for 2 hours at 37°C. After incubation, the optical density (OD) at 450 nm was measured with an auto-microplate reader (Infinite M200; Männedorf, Switzerland). To assess colony generation, cells were cultured in 6-well plates for 7 days, fixed with 4% formaldehyde for 20 minutes, and then stained with 1.0% crystal violet.

*Invasion assay*

Cell invasion was evaluated with a Transwell Matrigel (BD Biosciences, CA) assay. Transfected cells (200 mL) and complete medium (600
mL) were added to the upper and lower parts of the chamber, respectively. After a 48-hour incubation, non-invading cells in the upper chamber were removed with a cotton swab, whereas invading cells on the lower chamber were counted after fixation with 4% paraformaldehyde for 20 minutes, staining with crystal violet, and examination under a confocal microscope.

**Luciferase reporter assay**

A 24-hour cell culture was aliquoted into 24-well plates. Then, the cells were transfected with 5 ng of pRL-TK renilla plasmid (Promega, WI, USA) and 100 ng of pGL3-IGF2BP1-luciferase plasmid using Lipofectamine 2000 reagent according to the manufacturer’s instructions. The target genes of miR-372 were predicted by using TargetScan (www.targetscan.org). We identified LATS2 as a potential target of miR-372 by the presence of a miR-372 binding site in the 3’-UTR. Thus, the 3’-UTR of LATS2 was amplified by PCR and cloned into the pGL3-IGF2BP1 vector to generate pGL3-IGF2BP1-LATS2-WT. Then, the miR-372 binding site in LATS2 was mutated and cloned into the pGL3-IGF2BP1 vector to generate pGL3-IGF2BP1-LATS2-MT. These constructs were transfected into cells containing the luciferase plasmids. Forty-eight hours after transfection, luciferase activity and control signals were determined with the Reporter Assay Kit (Promega).

**Apoptosis assay**

The Annexin V-FITC Kit (Beckman Colter, CA) was used to assess apoptosis. Briefly, cells seeded in 6-well plates were transfected with a miR-372 inhibitor or negative control. Twenty-four hours after transfection, the cells were stained with PI and FITC-Annexin V and incubated in the dark for 15 minutes at room temperature. Then, cell death was analyzed by flow cytometry (FC), and the obtained data were analyzed with Cell Quest software. All experiments were performed in triplicate.

**Cell cycle analysis**

For the cell cycle analysis, cells were seeded into 6-well plates at the density of 2 x 10^5 cells per well and transfected with the miR-372 inhibitor or a negative control. Next, 48 hours after transfection, the cells were fixed with 70% ethanol and stained with 20 µg/mL PI. Then, the cell cycle distribution was analyzed by flow cytometry with a FACSCalibur (BD Biosciences, San Jose, CA). All experiments were performed in triplicate.

**Western blotting (WB)**

Total proteins were separated by SDS-PAGE, and then transferred to a PVDF membrane. The membrane was incubated with the primary antibodies and then with horseradish peroxi-
dase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). For western blotting, anti-LATS2 (ab135794; Abcam, Cambridge, MA, USA) and anti-β-actin (ab8229; Abcam) were used as the primary antibodies at a dilution of 1:1000.

Xenograft tumor growth

All animal care and experimentation were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Our research study protocol was approved by Hanzhong Central Hospital, China. BALB/c nude mice were obtained from the Experimental Animal Center of the Chinese Academy of Sciences in Shanghai. The mice were administered SKBr3 cells by subcutaneous injection in their flanks (6 mice per group). As soon as the volume of tumor reached ≥100 mm³, 200 pmol of miR-372 inhibitor or a scrambled oligo (negative control) was injected into the xenograft in several places once every other day for 14 days. Then, tumor development was observed. Tumor volume was calculated according to the following formula: greater diameter × smaller diameter²/2.

Statistical analysis

The results are presented as the mean ± standard deviation of at least three independent experiments. Student’s t-test or one-factor analysis of variance was used to evaluate the significance of differences with Prism version 5 (GraphPad Software, Inc.). The Kaplan-Meier method was used to compare survival between groups. A P value less than 0.05 was considered statistically significant.

Results

Mir-372 expression is highly upregulated in BC tissues and cell lines

miR-372 expression was evaluated in BC tissues and cell lines by qRT-PCR. The results
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Figure 4. MiR-372 directly targets at LATS2. A. The graph demonstrates how to build reporter plasmids of luciferase. B. Spearman’s correlation between LATS2 and miR-372 transcription in BC specimens of human. C. WB as well as qRT-PCR was applied to evaluation of LATS2 transcription and translation in SKBr3 and BT549 cells which was transfected with NC or miR-372 I. D. Verification of the bond between miR-372 and LATS2 in SKBr3 and BT549 cells was conducted by luciferase assay. Data are presented as mean ± SD. *, P < 0.05 compared with the control group.

showed marked upregulation of miR-372 in BC tissues when compared to the levels in surrounding non-tumor tissues (Figure 1A). Additionally, miR-372 expression was assessed in two BC cell lines, BT549 and SKBr3, as well as the MCF12A normal mammary epithelial cell line. The analysis showed that miR-372 expression in BT549 and SKBr3 BC cells was markedly higher than that in MCF12A normal cells (Figure 1B).

miR-372 downregulation inhibits the proliferation of BC cells in vitro and in vivo

To investigate the effect of miR-372 on BC, we determined whether miR-372 silencing was able to suppress BC cell proliferation. First, SKBr3 and BT549 cells were transiently transfected with the miR-372 inhibitor (miR-372 I) or a negative control (NC), and downregulation of miR-372 was confirmed by qPCR (Figure 2A). According to the results of the CCK-8 assay, cells transfected with miR-372 I showed remarkable suppression of cell growth compared to cells transfected with the NC (Figure 2B). Additionally, we explored whether miR-372 affected cell cycle progression in BC cells. As shown in Figure 2C, miR-372 downregulation reduced the proportion of SKBr3 and BT549 cells in S phase and noticeably enhanced the proportion of cells in G1 phase, suggesting that the inhibition of growth observed following miR-372 I transfection was probably related to G1-phase arrest. To explore the effect of miR-372 on breast tumor development, nude mice were administered a subcutaneous injection of SKBr3 cells in their flanks. When the tumor volume reached approximately 100 mm³, miR-372 I or the NC was injected into the xenograft tumor in several places once every other day for 14 days, and tumor development was monitored. As shown in Figure 2D and 2E, the growth of tumors injected with miR-372 I was retarded compared to the growth of tumors injected with NC. These data suggest that miR-372 down-regulation inhibited BC proliferation both in vitro and in vivo.
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miR-372 downregulation inhibits BC invasion and triggers apoptosis in BC cells

To determine whether miR-372 modulates BC invasion, we performed a Transwell invasion assay. As shown in Figure 3A, miR-372 downregulation remarkably reduced SKBr3 and BT549 invasion (Figure 3A). We also assessed apoptosis in BT549 and SKBr3 cells. The results showed that apoptosis was enhanced in miR-372 I-transfected cells compared to that in control-transfected cells (Figure 3B).

miR-372 directly targets LATS2

To explore the mechanism underlying miR-372-modulated induction of BC proliferation and invasion, the target of miR-372 was explored by using miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.targetscan.org/vert_71/). Among the identified targets, LATS2 was chosen for further evaluation. LATS2 plays an essential role in the inhibition of cancer progression and the modulation of invasion and proliferation [21, 22]. As shown in Figure 4A, the analysis identified a region complementary to miR-372 in the 3’-UTR of LATS2. We also noted that miR-372 levels were negatively related to LATS2 expression in BC tissues (Figure 4B). Based on the results of WB and qPCR, suppression of miR-372 enhanced LATS2 transcription and translation in SKBr3 and BT549 cells (Figure 5). LATS2 modulates miR-372 activity. (A) The expression of LATS2 in SKBr3 cells which underwent cotransfection with miR-372 I siRNA and LATS2 were determined by WB. Growth (B), distribution of cell cycle (C), invasion (D) and cell apoptosis (E) were shown in SKBr3 cells cotransfected with miR-372 I siRNA and LATS2. Data are presented as mean ± SD. *, P < 0.05 compared with the control group.
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It has been reported that miRNAs can function as tumor suppressors and promoters of many types of malignancies [11-14]. In our study, we explored the role of miR-372 in BC. We demonstrated that miR-372 was strongly upregulated in BC cells compared to the levels in normal mammary cells. Moreover, miR-372 expression was remarkably elevated in BC tissues compared to the levels in non-tumor tissues. In contrast, it was reported that miR-372 expression was lower in HCC than in normal tissue samples, and enhanced expression triggered by transfection with a miR-372 mimic suppressed the proliferation and invasion of HCC cells [15]. In addition, miR-372 expression levels were also reduced in renal cell carcinoma cells and tissues [16]. Consistent with the results of our study, miR-372 expression levels were increased in oral carcinoma and were related to worse clinical outcomes [17]. These results suggest that miR-372 might have distinct effects on different cancers.

In nasopharyngeal carcinoma, miR-372 induced CCA at S phase, but had no effect on apoptosis [18]. In our study, downregulation of miR-372 triggered G1/S arrest and apoptosis and suppressed cell proliferation and invasion in vitro. Consistent with the results of our study, downregulation of miR-372 suppressed glioma invasion and proliferation [19]. In contrast, ectopic expression of miR-372 suppressed HCC cell proliferation and invasion in vitro [15]. These results suggested that miR-372 is involved in complicated regulatory mechanisms with diverse effects on different cancers.

Figure 6. MiR-372 down-regulation suppresses PI3K/Akt pathway. A. SKBr3 and BT549 cells were transfected with NC or miR-372 I. The change of protein expression was determined by WB. B. The change of protein expression level in xenograft tumor tissues supplemented with miR-372 I and NC was determined by WB.

4C). Then, a luciferase reporter assay was performed. The results demonstrated that miR-372 I-transfected cells had noticeably higher luciferase activity, and mutation of the identified miR-372 binding sites remarkably attenuated the increase in luciferase activity induced by miR-372 I transfection (Figure 4D). These findings indicate that miR-372 directly targets LATS2.

MiR-372 modulates BC cell proliferation and invasion mediated by LATS2

To verify whether suppression of miR-372 could be modulated by changing LATS2 levels, we transfected SKBr3 cells with LATS2 and miR-372 I siRNAs. WB verified that LATS2 siRNA remarkably decreased LATS2 expression in cells that were transfected with miR-372 I (Figure 5A). The results of a CCK-8 assay demonstrated that the cell growth suppression triggered by miR-372 I could be partially reversed by transfection with LATS2 siRNA (Figure 5B). In LATS2 siRNA-transfected cells, the G1/S arrest triggered by miR-372 suppression was also attenuated (Figure 5C). Therefore, the effects of miR-372 I on cell apoptosis and invasion were counteracted by LATS2 siRNA (Figure 5D and 5E), which suggests that miR-372 modulated cell invasion and proliferation via LATS2 signaling.

MiR-372 downregulation inhibits the PI3K/Akt pathway

Since LATS2 is able to modulate Akt phosphorylation [10], the effect of miR-372 I on the PI3K/Akt pathway was assessed. Our experiment revealed that the phosphorylation of various proteins in the PI3K/Akt pathway, such as Akt, mTOR, and P70S6K, was reduced in cells transfected with miR-372 I (Figure 6A). In addition, the levels of LATS2 and the abovementioned PI3K/Akt pathway members were also decreased in xenograft tumor specimens treated with miR-372 I (Figure 6B). These data showed that miR-372 is a modulator of the PI3K/Akt pathway.

Discussion

It has been reported that miRNAs can function as tumor suppressors and promoters of many types of malignancies [11-14]. In our study, we explored the role of miR-372 in BC. We demonstrated that miR-372 was strongly upregulated in BC cells compared to the levels in normal mammary cells. Moreover, miR-372 expression was remarkably elevated in BC tissues compared to the levels in non-tumor tissues. In contrast, it was reported that miR-372 expression was lower in HCC than in normal tissue samples, and enhanced expression triggered by transfection with a miR-372 mimic suppressed the proliferation and invasion of HCC cells [15]. In addition, miR-372 expression levels were also reduced in renal cell carcinoma cells and tissues [16]. Consistent with the results of our study, miR-372 expression levels were increased in oral carcinoma and were related to worse clinical outcomes [17]. These results suggest that miR-372 might have distinct effects on different cancers.
LATS2 is a novel member of the LATS family that is mapped to chromosome 13q11-12 [7]. It was reported that LATS2 remarkably reduced the oncogenic activity of miR-25 in ovarian cancer [20]. In addition, downregulation of LATS2 suppressed NSCLC migration and growth, and LATS2 expression was decreased in NSCLC tissues [21]. We showed that miR-372 directly targets LATS2 and that there is a negative correlation between LATS2 expression and miR-372 expression. We also demonstrated that miR-372 regulates BC cell proliferation and invasion by targeting LATS2. This is the first report showing that LATS2 is targeted by miR-372. Li Yunfang et al. reported that LATS2 inhibited G1/S transition [22]. In our study, we showed, by WB, that downregulation of miR-372 suppressed the PI3K/Akt pathway. Phosphorylation of the majority of the PI3K/Akt pathway components mTOR, Akt, and P70S6K was reduced in cells transfected with miR-372 I. Consistent with the results of our study, downregulation of miR-372 also suppressed the PI3K/Akt signaling pathway in gliomas [19].

In conclusion, our findings demonstrated excessive expression of miR-372 in BC tissue samples. We also showed that miR-372 suppression inhibited BC cell proliferation and invasion by targeting LATS2, suggesting miR-372 suppression as a promising target for BC prevention and treatment.

Disclosure of conflict of interest

None.

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