Suppressive role of miR-592 in breast cancer by repressing TGF-β2

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Abstract. The function of miR-592 has been investigated in many types of cancer, however its roles in breast cancer remain unclear. We therefore investigated the biological function and underlying mechanism of miR-592 in breast cancer. In the present study, a marked downregulation of miR-592 was observed in breast cancer tissues and cell lines compared to the matched adjacent non-tumor tissues and normal breast cell line. Statistical analysis revealed that decreased miR-592 was negatively associated with advanced clinical stage, distant metastasis and lymph node metastases. Function analysis demonstrated that overexpression of miR-592 significantly inhibited cell proliferation, clone formation, migration and invasion in breast cancer cells in vitro, as well as suppressed tumor growth in vivo. Furthermore, transforming growth factor β-2 (TGFβ-2), a known oncogene, was identified as a direct target of miR-592, and its mRNA expression level was inversely correlated with the expression level of miR-592 in human breast cancer specimens. Restoration of TGFβ-2 expression rescued the inhibitory effect in breast cancer cells caused by miR-592. Collectively, these data suggest that miR-592 may exert its suppressive role in breast cancer, at least in part, by targeting TGFβ-2, and that miR-592 may be a novel target for breast cancer treatment.

Introduction

Breast cancer (BC) is the most common malignant tumor among women worldwide in the last decade (1). Although a large number of molecules have been identified as indicators in BC with the development of modern technology, the precise molecular mechanism and pathological process underlying growth and metastasis are poorly understood (2,3). Therefore, there is a continuing need to explore and understand the mechanism regulating BC initiation and progression to gain more effective and efficient therapeutic results for this disease.

MicroRNAs (miRNAs) are a group of small, endogenous, non-coding RNAs which function as the regulators of gene expression by binding to the 3'-untranslated region (3'-UTR) of their target genes (4). It has been demonstrated that miRNAs are involved in many biological processes, such as cell growth and survival, apoptosis, autophagy, migration, stem cell self-renewal and drug sensitivity (5). Experimental and clinical studies have shown that aberrations in miRNA expression are associated with tumorigenesis and cancer metastasis as tumor suppressors or oncogenes (6,7). Numerous miRNAs have been identified to play crucial roles in BC initiation and development (8,9).

In the present study, we focused our research on miRNA, miR-592, since it was identified as an important regulator in tumorigenesis and it may act as a tumor suppressor or an oncogene in different types of cancer (10–16). Previous studies have revealed that miR-592 was dysregulated in BC (17). However, the biological roles and underlying mechanism of miR-592 in BC remain unclear. Therefore, the aim of the present study was to investigate the biological function and the potential mechanisms of miR-592 in BC. We demonstrated that miR-592 was downregulated in both BC tissues and cell lines, and that miR-592 inhibited proliferation, migration and invasion in vitro, and suppressed tumor growth in vivo through targeting of TGFβ-2. These findings contribute to elucidate the functions of miR-592 and its underlying mechanism in BC progression.

Materials and methods

Patients and tissue samples. Primary human BC and their matched normal adjacent tissues were obtained from 56 BC patients who underwent surgery at The First Hospital of Jilin University (Changchun, China) from July 2014 to July 2016. Patients with incomplete clinical data collection or lack of clinical data or patients who had undergone chemotherapy, radiotherapy or any other treatment before surgery were excluded in the present study. All samples were immediately snap-frozen following surgery in liquid nitrogen and stored at -80°C until RNA extraction. All of the samples and clinical information were harvested after the patients provided written
informed consent which was approved by the Institutional Ethics Committees of Jilin University.

**Cell culture.** The non-cancerous human mammary epithelial cell line: MCF-10A, and 4 human BC cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen Corp., Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA) and antibiotics (100 U/ml penicillin or 100 µg/ml streptomycin sulfate) and 20 ng/ml epidermal growth factor (EGF), 0.1 mg/ml cholera toxin (CT), and 10 mg/ml insulin. The BC cell lines were all incubated in DMEM which contained 10% FBS, 100 U/ml penicillin plus 100 µg/ml streptomycin. All cells were grown under a humidified incubator with 5% CO₂ at 37°C. Other media supplies were obtained from Sigma-Aldrich (St. Louis, MO, USA).

miRNAs, plasmids and transfection. The miR-592 mimic and its negative control (miR-NC) oligonucleotides were obtained from GenePharma Co., Ltd. (Shanghai, China). TGF-β2-overexpressed plasmid was granted from Dr Tao Peng (Jilin University). Transfection was performed using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the transfection efficiencies were determined using qRT-PCR or western blotting.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR).** Total RNA including miRNAs were extracted from tissues and cultured cells using miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentrations of RNA were detected by NanoDrop 2000 (hermo Fisher Scientific, Inc., Waltham, MA, USA). The miRNA reverse transcription was performed according to the instructions of the OneStep PrimeScript® miRNA cDNA Synthesis kit, and then was quantified using SYBR Premix Ex Taq (both from Takara Biotechnology Ltd., Dalian, China) on an ABI 7900 Sequence Detection System (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. The primers of miR-592 and U6 were obtained from Applied Biosystems (Foster City, CA, USA). The real-time PCR for detection of TGF-β2 mRNA was performed according to the instructions of the OneStep SYBR® PrimeScript® PLUS RT-PCR kit (Takara Biotechnology Ltd.). The primers for TGF-β2 and GAPDH used in the present study were previously described (18). GAPDH/U6 were used as internal controls, and the relative expression of the target genes were calculated using the 2^ΔΔCt method.

**MTT assay.** Transfected BC cells were placed into a 96-well plate, at a density of 5x10^3 cells/well. After 24, 48, 72 and 96 h of culture in a CO₂ incubator at 37°C, the proliferation of BC cells was detected by MTT assay as previously described (18). The optical density (OD) at 570 nm of each well was assessed using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

**Colony formation assay.** The clonogenic survival assay was used to investigate the colony formation ability. Briefly, the transfected cells (1x10^3 cells/well) were plated into 6-well plates, and allowed to attach for 24 h. After being washed with phosphate-buffered saline (PBS), the cells were incubated for 10 days at 37°C in a humidified incubator. Finally, the images of the produced colonies were captured and counted under a light microscope (Olympus, Tokyo, Japan) after being fixed with 10% formaldehyde for 30 min and staining in 0.1% crystal violet solution (both from Sigma-Aldrich) for 10 min.

**Wound healing assay.** Transfected cells were incubated into a 6-well plate at 37°C. When the cells fully covered the plate bottom, the confluent monolayer in each well was created with a sterilized tip, ensuring that the width of each line was same. Following a 24-h incubation at 37°C, the cells were washed 3 times with PBS to remove any cell debris caused by the scratches. Images were captured at 0 and 24 h with an Olympus Inverted Microscope at 6 visual fields. The healing rate was calculated with the ImageTool software (Bechtel Nevada, Los Alamos Operations, USA).

**Transwell assay.** A cell invasion assay was performed using 24-well Transwell chambers (8-µm pores; BD Biosciences, San Jose, CA, USA). In short, transfected cells (1x10^3) were seeded onto Transwell chambers with Matrigel in serum-free medium. DMEM containing 10% FBS was added to the lower chamber as the chemoattractant. After incubation at 37°C for 48 h, the noninvading cells were removed with cotton swabs, whereas the invasive cells at the bottom of the membrane were fixed with 90% alcohol and stained with 0.1% crystal violet for 5 min. The invaded cells were counted and photographed using an Olympus Inverted Microscope in at least 5 randomly selected fields.

**Dual-luciferase assay.** A wild-type TGF-β2-3′-UTR, containing the miR-592 binding sites in the 3′-UTR region and a mutant-type TGF-β2 with a mutant sequence on the miR-592 binding site were amplified from human breast cDNA using PCR, and incorporated into the downstream of the firefly luciferase gene of a the pGL3-control vector (Promega, Madison, WI, USA). For luciferase assays, 1x10^5 cells were plated in 24-well plates and cultured for 24 h, then the cells were transfected with 100 ng wild-type or mutant-type reporter constructs, and 50 nM miR-592 mimic or the miR-NC with Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. The luciferase activity was assessed 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was then normalized to the corresponding Renilla luciferase activity.

**Western blotting.** Total protein was extracted from cultured cells or tissues with RIPA lysis buffer containing a protease inhibitor (Wuhan Boster Biotechnology Co., Ltd., Wuhan, China) on ice for 30 min. Total protein concentrations were assessed using a bicinchoninic acid (BCA) kit (Wuhan Boster Biotechnology Co., Ltd.). After the addition of the loading buffer, the extracted proteins were heated at 95°C for 10 min. A total of 30 µg loading buffer were separated
using 10% SDS polyacrylamide gels (SDS-PAGE), and then were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Laboratories Inc.). The membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature, following incubation with a primary antibody against TGF-β2 (diluted at 1:1,000) or GAPDH (diluted at 1:5,000) (both from Santa Cruz Biotechnology, Inc., Cruz CA, USA) at 4°C overnight. The membranes were incubated with horseradish peroxidase (HRP)-conjugated corresponding second antibodies (diluted at 1:6,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The protein bands were detected using the SuperSignal West Pico ECL chemiluminescence kit (Thermo Scientific, Rockford, IL, USA) and exposed on an X-ray film. The protein levels were normalized to GAPDH.

Xenograft assays in nude mice. Four-week-old BALB/c female nude mice were purchased from the Experiments Animal Center of Changchun Biological Institute (Changchun, China), and kept under specific pathogen-free (SPF) conditions. To establish the BC xenografts, ~2x10⁶ MCF-7 cells were subcutaneously inoculated into the right flank of each nude mouse, respectively. On day 10, when tumors reached ~100 mm³, mice were randomly divided into control and treatment groups (n=10/group). Then, the mice were intratumorally injected with miR-592 mimic or miR-NC 3 times/week for 4 weeks. The tumor width and length were assessed every 7 days using a caliper. The tumor volume was monitored and calculated according to the formula: V (volume) = 1/2 x length x width². The mice were sacrificed at 28 days post-implantation. Xenograft tumors were excised, photographed, weighed and stored at -80°C until use.

Statistical analysis. All data were analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) and were expressed as the mean ± standard deviation (SD) from at least 3 independent experiments. The t-test was used for comparisons between 2 groups. One-way ANOVA was applied for comparisons between multiple groups. The correlations between miR-592 and TGF-β2 were analyzed in BC tissues using Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant result.

Results

Expression level of miR-592 in BC tissues and cell lines. To investigate the expression of miR-592 in BC, firstly, 56-paired BC and adjacent normal breast tissues were detected in the present study. As shown in Fig. 1A, the expression of miR-592 was significantly downregulated in BC samples compared to matched adjacent normal tissues (P<0.01). The correlation between the expression levels of miR-592 and the clinicopathological characteristics were also investigated. As shown in Table I, decreased miR-592 was significantly associated with clinical stage, distant and lymph node metastases (P<0.01; Table I), whereas no statistical difference was found in the correlation between miR-592 expression and age,
miR-592 inhibits the proliferation and colony formation of BC cells. In order to explore the involvement of miR-592 in BC development and progression, miR-592 mimic or miR-NC were transfected into MCF-7 cells to restore its expression level as assessed by qPCR (Fig. 2A). The effect of miR-592 on cell proliferation was analyzed using an MTT assay. As shown in Fig. 2B, the cells transfected with the miR-592 mimic significantly inhibited cell proliferation compared to cells transfected with miR-NC from day 2 until 4, in a time-dependent manner. Consistent with these results, restored expression of miR-592 also significantly inhibited colony formation in MCF-7 cells (Fig. 2C).

miR-592 inhibits the migration and invasion of BC cells. To investigate the effect of miR-592 on the migration and invasion ability of BC cells, wound healing and Transwell invasion assays were performed, respectively. It was found that restoration of miR-592 expression significantly inhibited cell migration and invasion capabilities in MCF-7 cells (Fig. 3A and B), suggesting that miR-592 was able to inhibit the migration and invasion of BC cells in vitro.

TGF-β2 as the target gene of miR-592 in BC cells. TargetScan Human (http://www.targetscan.org) predicted that TGF-β2 is the target gene of the miR-592 (Fig. 4A). To investigate whether TGF-β2 is the target gene of miR-592, we transfected the miR-592 mimic and miR-NC into MCF-7 cells. Consequently, TGF-β2 expression at both the mRNA level and protein level was significantly suppressed in cells transfected with the miR-592 mimic compared to cells transfected with miR-NC (Fig. 4B and C). Meanwhile, luciferase reporter assay further revealed that MCF-7 cells transfected with the miR-592 mimic significantly inhibited the wild-type-TGF-β2-3'-UTR reporter activity (P<0.01; Fig. 4D), while it had no inhibitory effect on the mutant-type-TGF-β2-3'-UTR reporter activity (Fig. 4D). These data revealed that TGF-β2 was a target of miR-592 in BC.

TGF-β2 is upregulated, and is inversely correlated with miR-592 expression in BC tissues. We also evaluated the expression of TGF-β2 in 56-paired BC and adjacent normal breast tissues by qRT-PCR. The data revealed that the average level of TGF-β2 mRNA was significantly increased in BC tissues when compared with adjacent normal tissues. Moreover, TGF-β2 mRNA levels in BC cases were inversely correlated with miR-592 expression (r=-0.529; P<0.0001). In addition, TGF-β2 expression was upregulated at the mRNA level and protein level in 4 BC cell lines compared to non-malignant breast epithelial cell line, MCF-10A (Fig. 5C and D).

Tumor-suppressing function of miR-592 rescued by TGF-β2. To investigate the functional relevance of TGF-β2 targeting by miR-592 in BC, we assessed whether TGF-β2 overexpression and tumorsize (P>0.05). Furthermore, we assessed miR-592 expression levels in 4 BC cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) by quantitative real-time PCR (qPCR). As shown in Fig. 1B, the expression of miR-592 was found to be downregulated in all BC cell lines in contrast to the expression level of the non-malignant breast epithelial cell line, MCF-10A. Additionally, compared with the other BC cell lines, miR-592 was expressed the lowest in the MCF-7 cell line (Fig. 1B), and was selected for subsequent study.
rescued the inhibitory effects of miR-592 on BC cell proliferation, migration and invasion. We restored TGF-β2 expression by transfection with TGF-β2 overexpression plasmid in miR-592 mimic transfected MCF-7 cells (Fig. 6A and B). In addition, we also found that the forced expression of TGF-β2 rescued the inhibition of cell proliferation, colony formation,
Figure 5. TGF-β2 is upregulated, and inversely correlated with miR-592 expression in BC tissues. (A) The expression levels of TGF-β2 in 56 pairs of BC and adjacent normal tissues. GAPDH was used as an internal control. (B) The correlation of the expression levels of TGF-β2 and miR-592 were analyzed in BC tissues by Pearson’s correlation assay (n=56). (C and D) The TGF-β2 expression at the mRNA and protein levels was determined in 4 BC cell lines (MCF-7, MDA-MB-231, BT-549 and MDA-MB-453) and normal breast cell line MCF-10A. GAPDH was used as an internal control; **P<0.01.

Figure 6. Tumor-suppressing function of miR-592 rescued by TGF-β2. (A and B) TGF-β2 expression at the mRNA level and protein level was determined in MCF-7 cells transfected with the miR-592 mimic or miR-NC and with/without overexpression of the TGF-β2 plasmid. GAPDH served as the loading control. (C-F) Cell proliferation, colony formation, migration and invasion were analyzed in MCF-7 cells transfected with the miR-592 mimic or miR-NC and with/without overexpression of the TGF-β2 plasmid; *P<0.05, **P<0.01.
migration and invasion in the presence of the miR-592 mimic in MCF-7 cells (Fig. 6C-F). These findings inferred that miR-592 exerted its suppressive roles in BC by repressing TGF-β2.

miR-592 suppresses tumor growth in vivo. Finally, BC nude mice were set-up to investigate the role of miR-592 on cell growth in vivo. It was found that the tumor growth was slower in the MCF-7/miR-592 group compared to the MCF-7/miR-NC group (Fig. 7A). The mice were sacrificed at 28 days post-implantation, and the tumor tissues were stripped and weighed. We found that the size and weight of the tumors of the MCF-7/miR-592 group were significantly decreased compared to the MCF-7/miR-NC group (Fig. 7B and C). Furthermore, increased expression of miR-592 (Fig. 7D), and decreased TGF-β2 expression (Fig. 7E) were found in tumor tissues from the MCF-7/miR-592 and MCF-7/miR-NC groups. These data implied that miR-592 suppressed tumor growth in vivo.

Discussion

Accumulating studies have revealed that miRNAs plays negative or positive roles in the development and progression of breast cancer (BC) as tumor suppressors or oncogenes by inhibiting the expressions of genes related to tumorigenesis or metastatic dissemination (8,9). For example, Xu et al found that restoration of miR-154 significantly suppressed BC cell proliferation, migration and invasion, and increased cell arrest at the G1/G0 stage by suppressing E2F5 (19). Xia et al reported that miR-32 promoted proliferation and migration and suppressed apoptosis of BC cells by targeting FBXW7 (20). Kong et al demonstrated that miR-27a promoted the proliferation, migration and invasion of BC cells by targeting of the SFRP1 gene via Wnt/β-catenin signaling pathway (21). In the present study, we aimed to explore the role and underlying molecular mechanism of miR-592 in BC. We found that the expression level of miR-592 was downregulated in BC tissues and cell lines. Further function
analysis demonstrated that restoration of miR-592 significantly suppressed cell proliferation, colony formation, migration and invasion in vitro, as well as retarded tumor growth in vivo. These results indicated that miR-592 functioned as a tumor suppressor in BC.

TGF-β2 belongs to the transforming growth factor β (TGFβ) family which regulates a wide range of biological behaviors, such as embryogenesis, wound healing and tumorigenesis (22,23). In advanced stages of carcinogenesis, TGF-β2 can act as an oncogenic factor, and promote cancer cell proliferation, angiogenesis, invasion and metastasis (24,25). In BC, it has been demonstrated that TGF-β2 expression was upregulated in BC tissues compared to benign breast tissues (26), and that increased TGF-β2 expression promoted BC cell invasion and migration through the epithelial-mesenchymal transition (EMT) process (27). These data suggested that TGF-β2 may be a novel potential therapeutic target for BC.


