The Role of miR-640: A Potential Suppressor in Breast Cancer via Wnt7b/β-catenin Signaling Pathway

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In this study, we demonstrated that miR-640 is significantly downregulated in breast cancer (BC) tissues and cell lines. Overexpression of miR-640 inhibited the proliferation and migration of BC in vitro and in vivo, while depletion of miR-640 exhibited the opposite effect. Importantly, miR-640 could directly target Wnt7b, thereby regulating Wnt/β-catenin signaling pathway in BC. In conclusion, miR-640/Wnt7b suppresses BC cells tumorigenesis via Wnt/β-catenin signaling pathway, which might be novel targets for BC targeted therapy.

Keywords: miR-640, Wnt7b, β-catenin, Wnt/β-catenin pathway, breast cancer

INTRODUCTION

Breast cancer (BC) is well recognized as the most frequently diagnosed cancer in women worldwide with high incidence and mortality rates (1). Despite major breakthroughs in therapy strategies and therapeutic modalities of BC, the mortality rates of BC remain high due to metastasis and recurrence. Thus studies focusing on the mechanism of pathogenesis and metastasis of BC as well as new prognostic markers for precise are urgently needed.

Wnt signaling pathway, an important extracellular pathway, participates in a large set of cellular processes, including cell proliferation, differentiation, migration and apoptosis (2). The Wnt signaling pathway mainly comprises canonical Wnt pathway and noncanonical Wnt pathway according to whether β-catenin is affected (3). The canonical Wnt pathway is highly evolutionary conserved and mainly involves β-catenin. Previous studies have shown that aberrant activation of Wnt/β-catenin signaling pathway has a critical role in oncogenesis and development of various cancers, including BC (4, 5). Several Wnt proteins, such as Wnt1 (6, 7), Wnt3a (8), Wnt7a (9), Wnt7b (10) and Wnt9a (11) have been reported to initiate canonical Wnt/β-catenin signaling by combining with Frizzled (FZD) or low-density-lipoprotein receptor-related proteins 5/6 (LRP5/6), leading to the disassembly of the β-catenin destruction complex and nuclear translocation of β-catenin.
β-catenin. Then β-catenin could combine with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), thereby promoting transcription of Wnt target genes (3, 12, 13).

Wnt7b is highly-expressed in many cancers and aberrant Wnt7b expression contributes to the pathogenesis of several cancers, such as pancreatic adenocarcinoma, bladder cancer and osteosarcoma (10, 14, 15) (Figure S1). More importantly, high expression of Wnt7b is associated with aggressive clinicopathologic features and poor clinical outcome of BC patients (16), indicating it an independent prognostic biomarker in BC.

MicroRNAs (miRNAs) are a group of non-coding small RNA molecules that inhibit the expression of target mRNAs post-transcriptionally (17). Multiple miRNAs or miRNA families/clusters have been recognized play vital roles in BC. For example, miR-424 plays an anti-oncogenic role in BC by inhibiting CDK1 clusters have been recognized play vital roles in BC. For example, miR-424 plays an anti-oncogenic role in BC by inhibiting CDK1 (18). MiR-135b could promote BC cell growth and disrupt the cell cycle by regulating LATS2 (19). Recent studies indicate that miR-640 could aggravate intervertebral disc degeneration via NF-κB and WNT signaling pathway (20). In addition, miR-640 suppresses the progression of HCC via HIF-1α signaling pathway (21). However, the role of miR-640 in BC is still unknown.

In this study, we found miR-640 was downregulated in BC tissues and cell lines. In addition, miR-640 plays an anti-oncogenic role in vitro and in vivo by inhibiting BC cells proliferation and migration. More importantly, we demonstrated that Wnt7b is a direct target of miR-640, so that miR-640 could act as a suppressor in BC via canonical Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS

Clinical Tissue Samples

50 tumor tissues and their adjacent normal tissues were collected from BC patients who underwent operation in the Department of Breast and Thyroid Surgery of Shanghai Tenth People’s Hospital of Tongji University (Shanghai, China). Patients receiving chemotherapy or radiotherapy before surgery were excluded. This study was approved by Institutional Ethics Committees of Shanghai Tenth People’s Hospital. We have obtained informed consent from all patients. All tissue specimens were snap-frozen in liquid nitrogen immediately for further use.

Cell Culture and Transfection

We obtained the human BC cell lines MDA-MB-231, BT549, MCF-7, SKBR3 and normal breast epithelial cell line MCF-10A from Chinese Academy of Sciences (Shanghai, China). All BC cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Enprompse, China), and mycoplasma elimination reagent (Yeasen, China). MCF-10A were cultured in Mammary Epithelial Basal Medium (MEBM) (Cambrex, USA). All these cells were cultured at 37°C with 5% CO₂. MiR-640 mimics, miR-640 inhibitor and non-specific miR-negative control (miR-640-NC) oligo were purchased from RiboBio (Guangzhou, China). The specific siRNA of Wnt7b (si-Wnt7b) and negative control (si-NC) were purchased from General (Shanghai, China). Hieff Trans™ Liposomal Transfection Reagent (Yeasen, China) was used for transfection according to the protocols.

RNA Extraction and RT-qPCR

We extracted total RNA from frozen tissues and cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA was assessed by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). We conducted quantitative real-time polymerase chain reaction (RT-qPCR) by using the Hieff® qPCR SYBR® Green Master Mix (Yeasen, China). Primer sequences were designed and synthesized by RiboBio (Guangzhou, China). Expression of miRNAs and miRNAs were assessed by threshold cycle (CT) values and analyzed using the 2−ΔΔCt method. U6 and β-actin were used as internal control. Primers used in this study were shown in Table S1.

MTT Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell proliferation. Cells were seeded in 96-well plates at 1×10⁵ cells/ml after 24h transfection. 1, 2, 3, 4, 5 day later, 20 μl MTT (at a concentration of 5 mg/ml; Sigma Aldrich) was added per well, and cells were cultured at 37°C with 5% CO₂ for 4-6h. 150 μl DMSO was added after the discarding supernatant. The OD 490 nm optical density was detected by a microplate reader (BioTek, USA).

Colony Formation Assay

Transfected BC cells were seeded into 6-well plates (1000 cells per well). After 2 weeks, cell colonies were gently washed with cold 1 x PBS, fixed with 75% ethanol and stained with 0.1% crystaline purple. Then colonies were photographed and counted by Image J.

Wound Healing Assay

Transfected BC cells were seeded in 12-well plates. When the cells reached more than 90% confluent, we produced a scratch by using a 200-μl-pipette tip over the surface of cells. Then cells were cultured with DMEM medium with 2% FBS. The scratch area was observed and measured under the microscope (Leica Microsystems, Mannheim, Germany) at 0h and 24h. Image J was used to measure the scratch area.

Migration Assay

We used transwell chambers (Corning, Inc., Lowell, MA, USA) to measure the migration ability of MDA-MB-231 cells in 24-well plates. Transfected cells were placed in the upper chamber with 200 μl serum-free medium and medium with 10% FBS was added in the lower chamber. 14h later, noninvasive cells in the upper chamber were removed by cotton swabs and invasive cells on the opposite side were fixed with 95% ethanol for 10 min, stained with 0.1% crystal violet for 10 min. Pictures of invasive
cells were taken with a microscope (Leica Microsystems, Mannheim, Germany) and migrated cells were counted in 3 randomly-selected fields.

**Luciferase Reporter Assay**
Wild-type and mutant-type reporter plasmids of Wnt7b 3’-UTR were designed and synthesized by IBSBio (Shanghai, China). Two reporter plasmids were co-transfected with miR-640 mimics or miR-640-NC into HEK293T cells. After 48h, luciferase activities were measured by the Dual-Luciferase® Reporter Assay kit (Yeasen, China).

**FISH Assay**
Specific probes for miR-640 were designed and synthesized by IBSBio (Shanghai, China). 4’,6-Diamidino-2-Phenylindole (DAPI) was used to stain cell nuclei. Ribó™ Fluorescent In Situ Hybridization Kit (Ribó, China) was used in FISH assay. Fluorescence microscope (Olympus BX53 Biological Microscope) was used to capture the images of cells.

**Western Blotting Assay**
Proteins were isolated with RIPA lysis buffer (Beyotime, Jiangsu, NE, USA) was used to visualize protein bands. NE, USA) was used to visualize protein bands. Proteins were isolated with RIPA lysis buffer (Beyotime, Jiangsu, NE, USA) was used to visualize protein bands. Proteins were isolated with RIPA lysis buffer (Beyotime, Jiangsu, NE, USA) was used to visualize protein bands. 4% SDS-PAGE was used to separate the proteins. The membranes were incubated in secondary antibodies for 1h at room temperature. Dilutions of all antibodies used in this study were 1:1000. Odyssey Infrared scanning system (Li-Cor, Lincoln, NE, USA) was used to visualize protein bands.

**Xenograft Tumor Assay**
Athymic nude mice (age, 4–6 weeks; weight, 18–22 g) were obtained from the laboratory animal center of Shanghai and maintained in specific pathogen-free (SPF) conditions. Nude mice were randomly divided into two groups of 4 each. Approximately 1 × 10⁶ MDA-MB-231 cells with stable expression of miR-640 or miR-640-NC were injected into the second mammary fat of the mice. The growth of tumors was monitored every 1 week according to the following formula: Volume (mm³) = 0.5 * width² * length. After 7 weeks, the mice were sacrificed and the tumors were collected. The animal procedure was approved by the ethics committee of Tongji University.

**Immunohistochemistry (IHC)**
IHC were performed on formalin-fixed, paraffin-embedded sections of fresh tumor tissue samples from the nude mice. The paraffin-embedded tissue was incubated with anti-Wnt7b antibody (Proteintech, USA) at 1:250 dilution to measure Wnt7b expression overnight at 4°C. Images were captured under a microscope (Leica Microsystems, Mannheim, Germany) at the appropriate magnification.

**Statistical Analysis**
The significance of differences between groups from three independent experiments was assessed by GraphPad Prism (GraphPad, CA, USA). All data were presented as the means ± standard deviation (SD). The Student’s t-test was used for comparison between groups and P-value < 0.05 was considered statistically significant.

**RESULTS**
**MiR-640 Was Down-Regulated in BC Cell Lines and Tissues**
We searched several cancer research databases for characterizing the expression of miR-640, but there is no related report over the expression of miR-640 in BC. Thus, we first assessed the expression of miR-640 by RT-qPCR in 50 pairs of BC tissues and adjacent normal tissues. Our results showed that the expression of miR-640 was significantly downregulated in BC tissues (35/50, 70%) (Figures 1A, B). Additionally, we found the expression of miR-640 was lower in BC cell lines than in MCF-10A, especially in basal-like cohort and luminal cohort (Figure 1C). Then, we performed FISH assay to detected the localization of miR-640 and revealed that miR-640 was mostly stained in cytoplasm of MDA-MB-231 and MCF-7 (Figure 1D). Results of subcellular fractionation further verified the above results (Figures 1E, F). To better verify the role of miR-640 in BC, we analyzed the relationship between the expression of miR-640 and the clinical pathological variables in 50 BC patients. As shown in Table 1, high expression of miR-640 was negatively associated with TNM stage, tumor size and distant metastasis, but had no correlation with age and lymph node metastasis.

**MiR-640 Suppressed Cell Proliferation of BC Cells**
The transfection efficiency of miR-640-mimics and miR-640-inhibitor were verified by RT-qPCR (Figures 2A, B). Then, the MTT assay and colony formation assay revealed that upregulation of miR-640 caused a significant decrease in BC cells viability relative to the control group (Figures 2C, F). Meanwhile, the expression of proliferation marker PCNA was inhibited by miR-640-mimics demonstrated by western blotting (Figures 2G, H). All results above indicated that miR-640 act as an anti-tumor miRNA in BC.

**MiR-640 Suppressed Cell Migration of BC Cells**
The wound healing assay and transwell migration assay were performed to determine whether miR-640 affect BC cells migration. The results showed that the scratched area healing rate of the miR-640-mimics group was smaller compared to miR-640-NC group after 24 hours (Figures 3A, B). Consistently, we found that elevated miR-640 decreased the number of cells that passed through the membranes of transwell chamber in MDA-MB-231 cell line via transwell migration assay (Figures 3E, F). The miR-640-inhibitor group showed the opposite results (Figures 3C, D, G, H). These results indicated that miR-640 inhibit the migration of MDA-MB-231 cells.

**Table 1**
| Clinical Pathological Variable | Expression of miR-640 |
|-------------------------------|----------------------|
| Age                           | Negative correlation |
| Lymph node metastasis         | Negative correlation |
| TNM stage                     | Negative correlation |
| Tumor size                    | Negative correlation |
| Distant metastasis            | Negative correlation |
Wnt7b Is a Direct Target of miR-640

Wnt7b was predicted to have a direct binding site with miR-640 according to Targetscan (Figure 4A). Thus, luciferase reporter assay was performed to verify that Wnt7b is the direct target gene of miR-640. We constructed Wnt7b 3′-UTRs plasmids containing wild-type and mutant-type miR-640 binding sites. Through co-transfection of luciferase reporter plasmids and miR-640-mimics or miR-640-NC, we verified that wild-type Wnt7b 3′-UTR luciferase activity was remarkably decreased upon miR-640-overexpression (Figure 4B). After confirming Wnt7b is a direct target of miR-640, we further validated the effects of miR-640 on Wnt7b. Wnt7b expression in miR-640-overexpression or miR-640-depletion MDA-MB-231 and MCF-7 cells were determined by RT-qPCR. The results showed that the mRNA level of Wnt7b was remarkably downregulated via miR-640-overexpression while upregulated via miR-640-depletion (Figures 4C). Consistently, the results of western blotting indicated that miR-640 overexpression reduced Wnt7b, β-catenin, C-myc, and cyclin D1 protein levels whereas increased Gsk-3β protein level (Figures 4D–F). The miR-640 depletion showed the opposite results. More importantly, miR-640 upregulation inhibited the expression of β-catenin in the nuclear fraction of BC cells (Figures 4G, H). The above results prompted us to explore whether miR-640/Wnt7b suppresses BC tumorigenesis via Wnt/β-catenin signaling pathway.

Depletion of Wnt7b Rescued the Effect of miR-640-Inhibitor on BC Cells

We designed rescue assays to further determine whether miR-640 affects the proliferation and migration of BC cells via Wnt7b. MDA-MB-231 and MCF-7 cells were co-transfected with miR-640-inhibitor and si-Wnt7b. Based on the results in Figure 5, the promotive effect of miR-640-inhibitor on BC cells was partially

**TABLE 1** The relationship between the expression of miR-640 and various clinicopathological variables.

| Patients Characteristics | Total | miR-640 expression | P value* |
|--------------------------|-------|--------------------|---------|
|                          |       | High (N=15) | Low (N=35) |       |
| Age                      |       | 20 | 8 | 12 | 0.2077 |
| <60                      |       | 30 | 7 | 23 |       |
| ≥60                      |       | 33 | 13 | 20 | 0.0434* |
| TNM stage                |       | 17 | 2 | 15 |       |
| I/II                     |       | 29 | 14 | 15 | 0.0009*** |
| Tumor size(cm)           |       | 21 | 1 | 20 |       |
| ≤2                       |       | 30 | 12 | 18 | 0.0588 |
| >2                       |       | 20 | 3 | 17 |       |
| Lymph node metastasis    |       | 42 | 15 | 27 | 0.0434* |
| negative                 |       | 3 | 8 | 0 | 0.0001 |

P value from Chi-square test (P < 0.05, *** P < 0.001.)
reversed by Wnt7b silence (Figures 5A–F). Consistently, the effect of miR-640-inhibitor on Wnt7b protein level was also partially reversed by si-Wnt7b (Figures 5G, H). More importantly, expression of β-catenin in the nuclear fraction showed the same changes with Wnt7b (Figures 5G, I).

**Overexpression of miR-640 Inhibited BC Growth In Vivo**

We established the MDA-MB-231 cell xenograft model to verify the effect of miR-640 in BC in vivo. As shown in Figure 6A, MDA-MB-231 cells were stably infected by lentiviral(lv-miR-640
MiR-640 suppressed cell migration of BC cells. (A–D) Wound healing assays were performed in MDA-MB-231 cell line treated with miR-640-mimics or miR-640-inhibitor (miR-NC as negative control). (D–H) Cell migration assays were performed in MDA-MB-231 cell line treated with miR-640-mimics or miR-640-inhibitor (miR-NC as negative control). ***p < 0.001.
Wnt7b is a direct target of miR-640. (A) Putative complementary site within miR-640 and Wnt7b predicted by bioinformatics analysis (TargetScan). (B) Luciferase reporter assay demonstrated that Wnt7b is a direct target of miR-640. (C) Wnt7b mRNA level was determined by RT-PCR in MDA-MB-231 and MCF-7 cells with different treatment. (D–F) Representative Western blotting and quantification of Wnt7b, β-catenin, Gsk-3β, Cyclin D1 and C-myc in MDA-MB-231 and MCF-7 cells with different treatment, β-actin was used as a control. (G, H) Representative Western blotting and quantification of β-catenin in nuclear fraction and cytoplasm fraction of BC cells with different treatment, LaminA was used as a control. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. “ns” means not statistically significant and represents P < 0.05.
FIGURE 5 | Depletion of Wnt7b rescued the effect of miR-640-inhibitor on BC cells. (A–D) Knockdown of Wnt7b partially reversed miR-640-inhibitor-induced promotion of proliferation in MDA-MB-231 and MCF-7 cells determined by MTT assay and colony assay. (E, F) Knockdown of Wnt7b partially reversed miR-640-inhibitor-induced promotion of migration in MDA-MB-231 and MCF-7 cells determined by transwell assay. (G–I) Western blotting analysis for Wnt7b/β-catenin protein level in MDA-MB-231 and MCF-7 cells. **p < 0.01, ***p < 0.001, ****p < 0.0001.
or lv- miR-640-NC). 7 weeks later, tumors from the nude mice were collected and measured. Obviously, tumors in the miR-640-overexpression group were significantly smaller than those in miR-640-NC group, indicating that tumor growth was significantly inhibited by the miR-640 (Figures 6B, C). Then proteins were extracted from mice tumors and western blotting results indicated that the protein level of Wnt7b decreased in the miR-640-overexpression group (Figure 6D). Moreover, IHC analysis further elucidated that tumors of miR-640-overexpression had lower Wnt7b expression (Figure 6E). Taking all results in vivo and in vitro together, we confirmed that miR-640/Wnt7b suppresses BC cells tumorigenesis via Wnt/β-catenin signaling pathway. The mechanism was generated in Figure 6F.
DISCUSSION

As a group of small non-coding RNAs, miRNAs play key roles in the gene regulatory network of tumors by binding to the 3’ untranslated region (3’-UTR) of target mRNAs to inhibit gene expression. In recent years, multiple studies have focused on the function and mechanism of miRNAs in BC, so that many miRNAs have been found related to the initiation and progression of BC. Additionally, miRNA-based therapies have already been used as vital strategies in BC (22). In recent years, several studies revealed that miR-640 participate in numerous different biological processes. MiR-640 could promote Kupffer cells inflammation via restraining LRPI and Wnt signaling pathway, indicating it a potential target for the therapy of acute liver injury in the future (23). MiR-640 participates in the process of sevoflurane-induced abnormal cognition via regulating ZFP91 (24). In addition, miR-640 plays a pivotal role in mediating the proangiogenic effect of H2S (25). However, there is no related reports over the role of miR-640 in the tumorigenesis and progression of BC. This study is the first to show that the expression of miR-640 in BC and the first to report the mechanism and clinical significance of miR-640 in BC.

In the present study, we first investigated the expression of miR-640 in 50-paired clinical BC tissues and adjacent normal tissues, and revealed that miR-640 expression is dramatically downregulated in BC. Of note, high expression of miR-640 was negatively associated with TNM stage, tumor size and distant metastasis of BC. Consistent with its expression pattern, overexpression of miR-640 could remarkably inhibit the proliferation and migration of BC in vitro and in vivo. Based on the expression level and function of miR-640 in BC, we further hypothesized that miR-640 could directly inhibit Wnt7b according to according to prediction from Targetscan.

Wnt7b, an important member of the Wnt proteins family, have been reported highly-expressed in many malignant tumors, including BC (26–28). Wnt7b could activate mTORC1 via PI3K/ AKT signaling pathway to promote bone formation (29). Moreover, Wnt7b promotes cancer cell androgen-independent growth by activating protein kinase C isozymes in advanced prostate cancer (26). Of note, Wnt7b have been reported facilitate several cancers tumorigenesis via regulating canonical Wnt/β-catenin signaling (28, 30, 31). To verify whether miR-640 could directly target Wnt7b, luciferase reporter assay was performed. Then, according to the results of RT-qPCR and western blotting, both miRNA and protein level of Wnt7b were negatively regulated by miR-640. Consistently, protein levels of total β-catenin, cyclinD1 and C-myc were negatively regulated by miR-640 while Gsk-3β showed opposite results. More importantly, miR-640 upregulation inhibited the expression of β-catenin in the nuclear fraction, indicating the inhibitory effect of miR-640 on canonical Wnt/β-catenin signaling pathway. Finally, the rescue experiment further verified miR-640 suppresses BC via Wnt7b/β-catenin signaling pathway.

In conclusion, our findings demonstrated that miR-640 is downregulated in BC tissues and cell lines, and is able to suppress BC tumorigenesis in vitro and in vivo through directly targeting Wnt7b and Wnt/β-catenin signaling pathway. Therefore, the miR-640/Wnt7b/β-catenin axis might be novel targets for BC targeted therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Tenth People’s Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Shanghai Tenth People’s Hospital.

AUTHOR CONTRIBUTIONS

CT, XW, and LF designed the research. CT and XW performed the research and analyzed results. XW wrote the paper. CJ, WZ, YY, XD, and XZ edited the manuscript and provided critical comments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.645682/full#supplementary-material

Supplementary Figure 1 | Expression of Wnt7b in BC (TCGA database).
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.