

MiR-27a-3p Targeting GSK3 β Promotes Triple-Negative Breast Cancer Proliferation and Migration Through Wnt/ β -Catenin Pathway

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Background: Dysregulation of microRNAs (miRNAs) was found to play crucial roles in varieties of cancers, which affect tumor proliferation and migration. MiR-27a-3p has been identified as a tumor-related miRNA in liver cancer, lung cancer, and colorectal cancer. However, the function of miR-27a-3p in triple-negative breast cancer (TNBC) and its possible molecular mechanisms have still not been elucidated.

Methods: QRT-PCR technique was used to detect the expression of miR-27a-3p in TNBC and normal breast cell lines or the effects of miR-27a-3p knockdown and overexpression in TNBC cell lines. Proliferation and migration were measured by CCK-8 method, colony formation, wound healing, and Transwell assays, respectively. Furthermore, we used a dual-luciferase reporter gene assay and Western blot analysis to identify GSK3 β as a target of miR-27a-3p.

Results: In this study, we found that miR-27a-3p expression was significantly elevated in TNBC cell lines. Database analysis suggested that TNBC patients with a high expression of miR-27a-3p have poorer overall survival possibilities. Overexpression of miR-27a-3p promotes TNBC cells proliferation, colony formation, and cell migration in vitro. Nevertheless, dual-luciferase reporter result showed that miR-27a-3p directly targeted the 3'-UTR regions of GSK3 β mRNA and negatively regulated its expression. Lastly, we demonstrated that miR-27a-3p inactivates Wnt/ β -catenin signaling pathway via targeting GSK3 β .

Conclusion: These results indicate that expression of miR-27a-3p was highly expressed in TNBC and promoted tumor progression through attenuating GSK3 β and may have a potential molecular-targeted strategy for TNBC therapy.

Keywords: triple-negative breast cancer, miR-27a-3p, GSK3 β , Wnt/ β -catenin pathway

Introduction

Breast cancer is one of the most common malignant tumors in women and the sixth leading cause of cancer-related death worldwide.¹ In 2017, breast cancer will be diagnosed in 12% of women in the United States over the course of their lifetimes and 9.6% of all deaths from breast cancer worldwide.² Triple-negative breast cancer (TNBC) accounts for 15% to 20% of newly diagnosed breast cancer cases and is known for its aggressive biological behavior and poor patient outcomes compared with hormone receptor-positive breast cancer.³ TNBC is defined as a subtype of breast cancer that does not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).⁴ Metastasis, recurrence, and high susceptibility to drug resistance are the main factors related to poor

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prognosis in patients with TNBC.⁵ Currently, due to the devoid of early detection biomarkers and clear therapeutic targets, TNBC patients are often diagnosed late with a high histological grade and do not benefit from hormonal or targeted therapies.⁶ Therefore, effective therapeutic approaches for TNBC are essential to improve outcomes for patients with this malignancy.

MicroRNAs (miRNAs) are small non-coding RNAs, 19–25 nucleotides in length, and these RNAs can bind to the 3'untranslated regions (3'UTRs) of their target mRNA to regulate gene expression at the posttranscriptional stages.^{7,8} MiRNAs exert a wide variety of fundamental biological processes in TNBC development including cell proliferation, invasion, and metastasis.⁹ Some of miRNAs are emerging as potential targets for diagnostic and therapeutic interventions in TNBC.^{10,11} Previous studies have shown that the expression of several miRNAs was dysregulated during the carcinogenesis and progression of TNBC, such as miR-335, miR-873, miR-483-3p, and miR-124.^{12–15} Abnormal expression of miRNAs affects the oncogenic behaviors of TNBC cells through play oncogenes or tumor suppressor roles.¹⁶ Therefore, it is important to understand abnormally expressed miRNAs in TNBC progression to realize potential therapeutic targets. The miR-27a-3p has been widely implicated in the tumorigenesis of a variety of cancers, the expression and function of miR-27a-3p in TNBC remain poorly understood.

The canonical Wnt signal pathway, also known as the Wnt/ β -catenin or the β -catenin/T-cell factor (TCF) pathway, controls many biological processes via signal transduction, including cell proliferation, cell fate determination, and organ formation.¹⁷ The key regulatory factor within Wnt pathway is GSK3 β .^{18,19} A large number of studies have reported that GSK3 β proteins play important roles during embryo development.^{20–22} Notably, recent studies show that GSK3 β act as oncogenes regulating cancer development in several types of cancers.^{18,21} In this study, we investigated the effect of GSK3 β on triple-negative breast cancer for the first time.

In our research, we confirmed that miR-27a-3p was overexpressed in TNBC cell lines. Elevated miR-27a-3p expression predicted a poor prognosis in TNBC patients. In vitro, miR-27a-3p overexpression promoted cell growth and migration. Furthermore, we demonstrated that miR-27a-3p promoted cell proliferation and migration by directly targeting GSK3 β and inactivated Wnt/ β -catenin signaling in TNBC. Together, these results implicated

that miR-27a-3p interference may be a potential method for TNBC therapy.

Materials and Methods

Cell Culture

Breast cancer cell lines (BT-549, MDA-MB-231, MDA-MB-468, MDA-MB-453) and immortalized human breast epithelial cell lines (MCF-10A, DU4475) were all obtained from the American Type Culture Collection (ATCC). BT-549, MDA-MB-231, MDA-MB-468, and MDA-MB-453 were cultured in recommended medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified incubator at 37°C with 5% CO₂. MCF-10A and DU4475 were cultured in complex including Mammary Epithelial Cell Growth Medium DMEM (Gibco, USA), 5% Horse serum (Gibco, USA), 20ng/mL EGF (Sigma, USA), 0.5 μ g/mL hydrocortisone (Thermo Fisher Scientific, USA), 10 μ g/mL insulin (Thermo Fisher Scientific, USA), 100ng/mL cholera toxin (Sigma, USA) in a humidified incubator at 37°C with 5% CO₂.

Cell Transfection with miRNA Mimics or Inhibitors

MiRNA NC, miR-27a-3p mimic, and miR-27a-3p inhibitor were purchased and biosynthesized from Ribobio (Guangzhou, China). The cells were seeded into 6-well plates and transfected with 100 nM oligonucleotides (miRNA NC, miR-27a-3p mimic, and miR-27a-3p inhibitor) using Lipofectamine 3000 reagent (Invitrogen, USA) in recommended concentrations according to the manufacturer's instructions.

RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA from cells was extracted using Trizol Reagent (Takara, Japan) and quantified with NanoDrop 2000 (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using PrimeScript™ RT Master Mix (Takara, Japan). Quantification of miRNA expression was performed using the Mir-X™ miRNA qRT-PCR TB Green™ Kit (Takara, Japan) on a Light Cycler® 480 II (Roche, Applied Science). Primers were synthesized by Ribobio (Guangzhou, China) and its sequences as follows: U6 snRNA forward, 5'-ATTG GAACGATACAGAGAAGATT-3' and reverse, 5'-GGA ACGCTTCACGAATTTG-3; miR-27a-3p, forward, 5'-TGCGGTTACAGTGGCTAAG-3' and reverse, 5'-CTC

AACTGGTGTCTGGA-3'. MiR-27a-3p expression level was normalized to the U6 small nuclear RNA level and calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Assay

BT-549 and MDA-MB-231 cells were seeded into 96-well plates at a density of 5000 cells per well and transfected with 100 nM oligonucleotides (miRNA NC, miR-27a-3p mimic, and miR-27a-3p inhibitor). Cells were incubated at the cell culture condition for 0, 24, 48, and 72 h. Cell Counting kit-8 (CCK-8; Sigma, USA) assay was performed according to the manufacturer's protocol. Finally, the absorbance of each sample was determined at a wavelength of 450 nm using microplate reader (Bio-Rad, USA). Each experiment was repeated three times.

Colony Formation Assay

BT-549 and MDA-MB-231 cells (1×10^3 cells/well) were seeded in 6-well plate with treatment of 100 nM oligonucleotides (miRNA NC, miR-27a-3p mimic and inhibitor) transfection and were then maintained in cell culture conditions for 14 days. Colonies were fixed using 4% paraformaldehyde, stained with 0.1% crystal violet. Cell colonies images were randomly taken by Nikon microscope. Cell colony formation was counted and analyzed.

Transwell Migration Assay

Cell migration assay was performed using Transwell chamber membranes (BD Biosciences, USA) in accordance with manufacturer's methods. Briefly, BT-549 and MDA-MB-231 cells were seeded in the upper chamber of the Transwell migration system after being transfected with 100 nM oligonucleotides (miRNA NC, miR-27a-3p mimic and inhibitor) and the lower chambers were filled with culture medium as chemoattractant and then stay for 2 days, migrated cells on the lower chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells were counted in five random fields under a light microscope. Each experiment was duplicated three times.

Wound Healing Assay

Cells were grown to 90% confluence on 6-well culture plates and were transfected with 100 nM miRNA NC or miR-27a-3p mimic and inhibitor. We Used a 10 μ L sterile pipette tip drew a straight line vertically at the center of each well. Images were captured immediately after wounding for indicated time points, and the healing was monitored by microscopy.

MiRNA Target Prediction and Luciferase Activity Assay

The miRNA target prediction and analysis were performed with the algorithms from miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>), TargetScan (<http://www.targetscan.org/vert72/>), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and RNA22 (<https://cm.jefferson.edu/rna22/>). We cloned the wild-type (WT) GSK3 β -3' untranslated region (UTR) or the GSK3 β -3'UTR with various miRNA-binding site mutations (Mut) into pGL3-Basic-Vector. Luciferase reporter assays were performed using a dual-specific luciferase assay kit (Promega, USA) by following manufacturer's protocol. The reporters were transfected into cells using Lipofectamine 3000 (Invitrogen, USA). The ratio of F-luc/R-luc was calculated to analyze its expression level. Experiments were performed in triplicates.

Western Blotting Analysis

Total cell lysates were prepared with a detergent lysis buffer. Western blots were performed as instructions. In short, cellular proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and were probed with antibodies against GSK3 β (1:2000, Abcam), β -Catenin, p- β -Catenin (1:2000, Abcam), C-Myc (1:2000, Sigma) and Vimentin (1:1000, CST). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (1:1000, Abcam) were used as a secondary antibody. GAPDH protein levels were determined by using the specific antibody (1:5000, Abcam) as a loading control for cells, ECL reagent (Tanon, China) was used to visualize the protein bands.

Statistical Analysis

All data were displayed from three independent experiments in triplicate. The data represent the mean \pm SD unless otherwise indicated. Data were analyzed by two-tailed unpaired Student's *t*-test between two groups and by One-Way ANOVA followed by Bonferroni test for multiple comparisons. Significance was considered $P < 0.05$. Statistical analyses were performed using GraphPad PRISM (version 8.0; Graph Pad Software).

Results

Upregulation of miR-27a-3p in TNBC Cells

MiR-27a-3p is upregulated in TNBC cell lines. To determine the expression patterns of miR-27a-3p in TNBC cells, we analyzed the expression of miR-27a-3p in four TNBC cell

lines: BT-549 (BT549), MDA-MB-231 (231), MDA-MB-468 (468), MDA-MB-453 (453), and normal human breast epithelial cell lines MCF-10A and DU4475 by Western blotting analysis. QRT-PCR results confirmed that the expression level of miR-27a-3p in all four TNBC cell lines was significantly increased than that in MCF-10A and DU4475 ($P < 0.05$; Figure 1A). Furthermore, analysis of TCGA survival data revealed that patients with high miR-27a-3p expression were associated with poor overall survival (Figure 1B). In total, miR-27a-3p might be associated with TNBC progression.

Effects of miR-27a-3p Expression on Cell Proliferation and Migration of TNBC

We confirmed the upregulation of miR-27a-3p expression in TNBC cells compared with normal human breast epithelial cells and supposed that miR-27a-3p may play an oncogenic role in TNBC. Therefore, we further explored the effects of miR-27a-3p on proliferation and migration of TNBC cells, we transfected miRNA NC (miR-NC), miR-27a-3p mimic, and miR-27a-3p inhibitor into the BT549 and 231 cell lines. The CCK-8 assay was used to measure cell proliferation and results showed that BT549 and 231 cell lines obviously increased the cell proliferation in miR-27a-3p mimic group compared to the miR-NC group. Meanwhile the proliferation of BT549 cells and 231 cell lines after being transfected with miR-27a-3p inhibitor was inversely suppressed compared with the miR-NC group (Figure 2A). Then, we further investigated the effects of miR-27a-3p on cell migration by wound healing assay and Transwell. Wound healing assay displayed that miR-27a-3p mimic obviously promoted wound closure, compared with

miR-NC in BT549 and 231 cell lines (Figure 2B). Transwell migration assays showed that miR-27a-3p mimic greatly promoted migration in BT549 cells, compared with that of the miR-NC, while inhibiting miR-27a-3p expression decreased the cell's migration ability compared to the miR-NC groups (Figure 2C). Similar trends were also observed in 231 cells. In addition, colony formation assay provided that the number of cell colonies in the miR-27a-3p mimic group was significantly higher than that in the miR-NC group, but the miR-27a-3p inhibitor groups presented the converse results (Figure 2D). All these results indicated that miR-27a-3p can promote cell proliferation and migration in TNBC cells.

GSK3 β is a Direct Target of miR-27a-3p in TNBC Cells

To identify novel miR-27a-3p target genes, we queried the different published prediction databases, including miRDB, miRWalk, PITA, TargetScan. Intriguingly, we identified a novel potential candidate as GSK3 β , which was predicted in on four databases (Figure 3A). To support our hypothesis that miR-27a-3p directly regulates GSK3 β expression through its 3'-untranslated region (UTR), we generated luciferase reporter plasmids, which harbored either WT or mutated-type (MT) miR-27a-3p binding sites within the 3'-UTR of GSK3 β (Figure 3B). The dual-luciferase reporter assay showed that miR-27a-3p inhibitor can notably augment luciferase activity in cells with WT-GSK3 β transfection, but did not affect those with MT-GSK3 β transfection (Figure 3C). What's more, miR-27a-3p inhibitor also significantly fortified luciferase activity with WT-GSK3 β transfection rather than

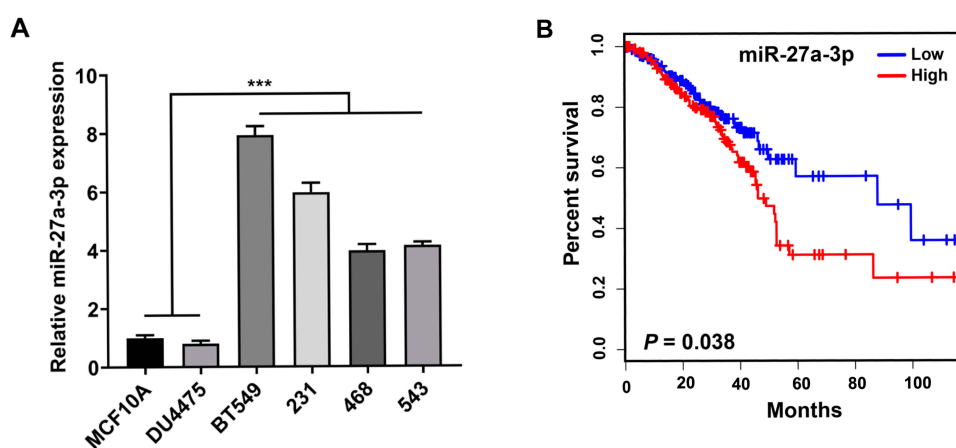


Figure 1 Overexpression of miR-27a-3p is associated with poor prognosis.

Notes: (A) MiR-27a-3p expression in TNBC cell lines and normal human breast epithelial cell lines. (B) MiR-27a-3p upregulation was significantly associated with shorter overall survival in patients with TNBC. Error bars, SD. *** $P < 0.001$.

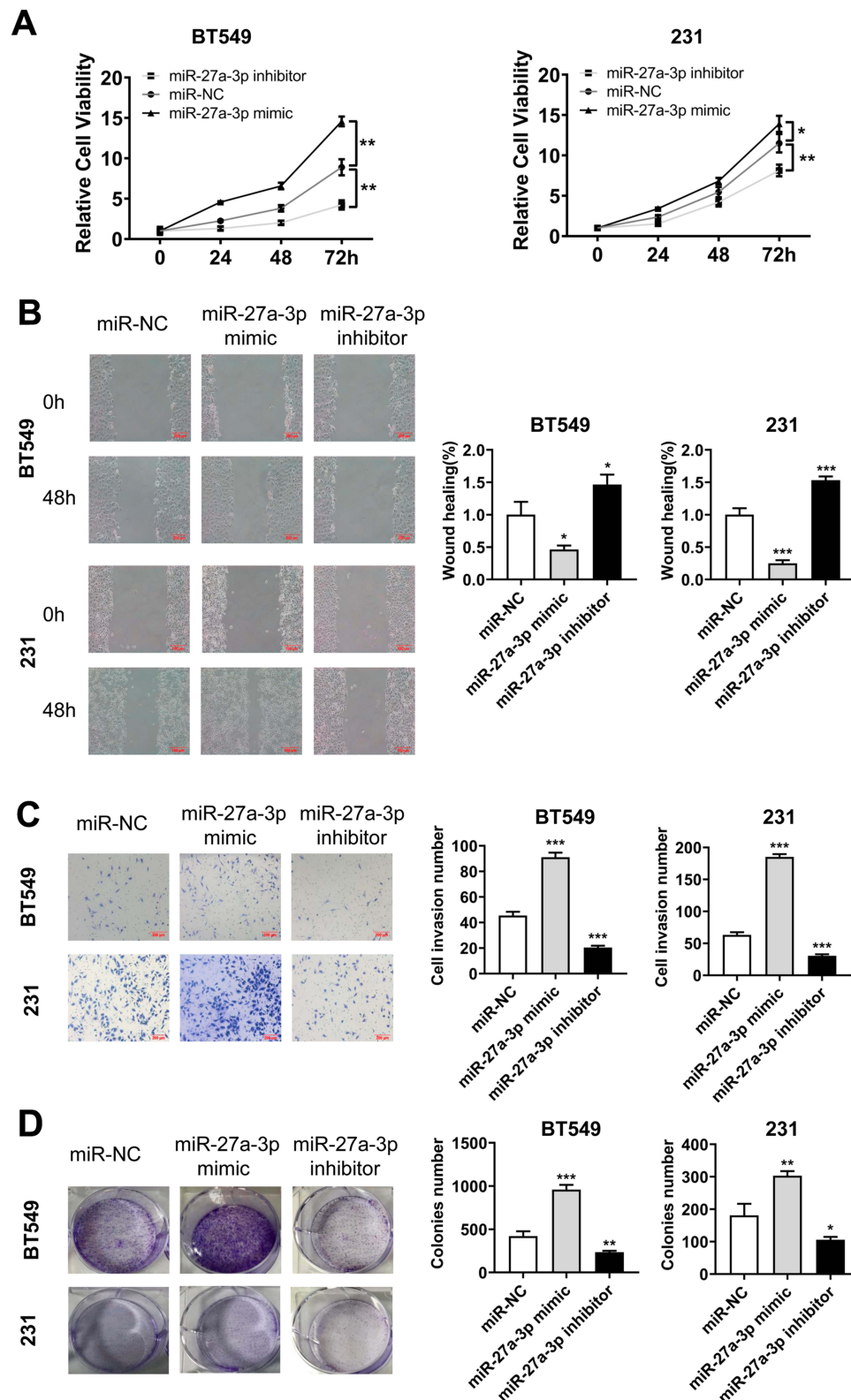


Figure 2 Overexpression of miR-27a-3p promoting TNBC cells proliferation, colony formation, and migration in vitro.

Notes: (A) Cell proliferation (CCK8 assay), (B) wound healing assay (right, quantitative analysis), (C) Transwell migration assays (quantitative analysis), and (D) colony formation assay (right, quantitative analysis) in the 231 and BT549 cell lines transfection with miR-NC, miR-27a-3p mimic, and miR-27a-3p inhibitor). Error bars, SD. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

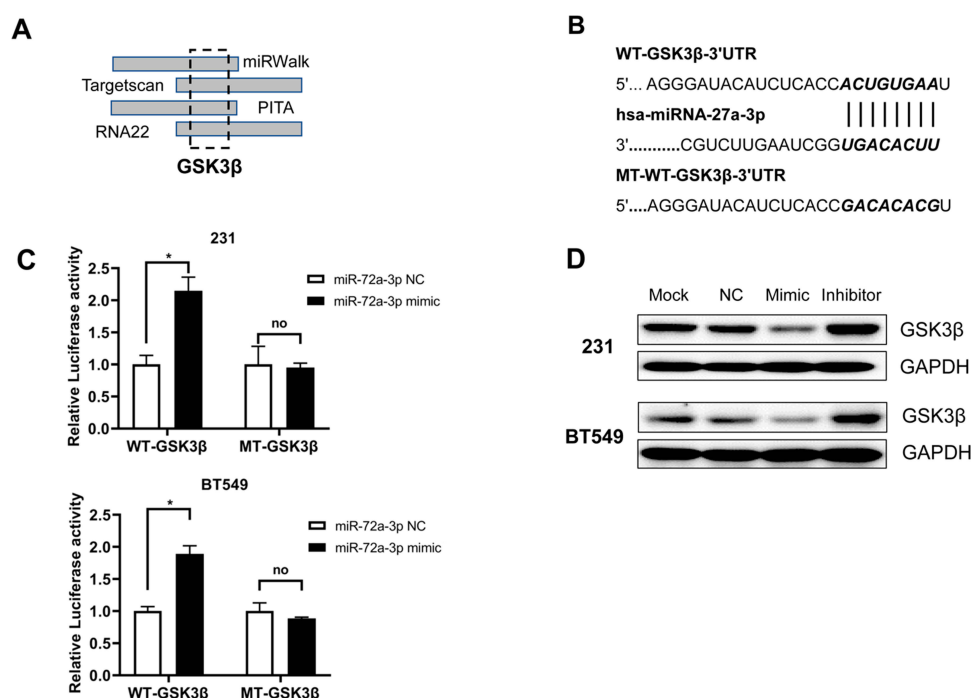


Figure 3 GSK3β was a target of miR-27a-3p in TNBC cells.

Notes: (A) The predicted miR-27a-3p binding site in the 3'-UTR of GSK3β. (B and C) Relative luciferase activity in 231 and BT549 cell lines with luciferase reporter vectors or synthetic miRNAs transfection. (D) The protein expression of GSK-3β is shown by Western blot in 231 and BT549 cell lines were transfected with miR-NC, miR-27a-3p mimic, and miR-27a-3p inhibitor for 24 h. Error bars, SD. * $P < 0.05$.

Abbreviation: no, not significant.

MT-GSK3β group in 231 and BT549 cell lines, suggesting that miR-27a-3p targets GSK3β via binding its 3'UTR. In addition, we overexpressed or inhibited miR-27a-3p in 231 and BT549 cell lines by using miR-27a-3p mimics or inhibitors. It is profoundly shown that GSK3β was consistently downregulated in miR-27a-3p overexpressing cells while upregulated in miR-27a-3p inhibition cells, suggesting GSK3β was a direct targeted gene of miR-27a-3p (Figure 3D). Taken together, we verified miR-27a-3p as a novel regulator of GSK3β through binding its transcript.

MiR-27a-3p Overexpression Inactivates the Wnt/β-Catenin Signaling Pathway

GSK3β has been reported to function as a negative regulator of Wnt/β-catenin pathway and phosphorylation of β-catenin is necessary for signal activation. Further, we detected the related genes of Wnt/β-catenin signaling pathway in BT549 and 231 cells after transfection with miR-NC, miR-27a-3p mimic, and miR-27a-3p inhibitor for 24 h by Western blotting, which was used to verify whether miR-27a-3p inactivate the pathway or not. Expectedly, it showed that the expression of C-Myc, Vimentin, and β-Catenin was totally increased and GSK3β, p-β-Catenin

was decreased with miR-27a-3p mimic treatment in both 231 and BT-549 cells (Figure 4). Thus, these results indicated that miR-27a-3p regulates negatively Wnt/β-catenin signal activation in TNBC cells.

Discussion

TNBC is a commonsense health burden in Asia, as the pathogenesis process is very complex, which results in the understanding of TNBC progression remains limited.²³ Despite advances in the treatment of TNBC in recent years, its prognosis has limitedly improved. In the past years, extensive studies have revealed miRNAs are critical regulators in TNBC progression.^{24,25} Aberrant expression of miRNAs may be used as prognosis prediction markers of therapeutic targets for TNBC.

It is well known that miRNAs can negatively regulate gene expression to perform various biological and pathological functions in posttranscriptional stage. Both GSK-3β and β-catenin are crucial regulators of Wnt/β-catenin signaling implicated in the progress of various tumors. Previous studies have pointed out that miR-27a-3p was recognized as a tumor-promoting miRNA in gastric cancer by targeting BTG2 or colorectal cancer by BTG1; it acts as a tumor

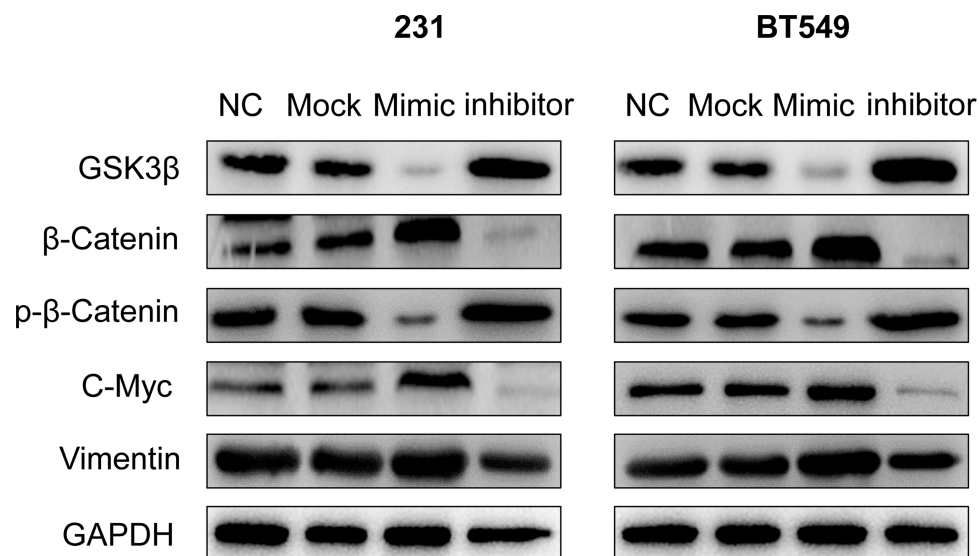


Figure 4 MiR-27a-3p overexpression inactivates the Wnt/β-catenin signaling pathway.

Notes: The protein expression of GSK-3β, β-catenin, p-β-catenin, C-Myc, and Vimentin is shown by Western blot in 231 and BT549 cell lines were transfected with miR-NC, miR-27a-3p mimic, and miR-27a-3p inhibitor for 24 h.

suppressor in non-small lung cancer via targeting HOXB8.^{26,27} However, miR-27a-3p participates in the progression of TNBC remains unknown. In our current study, we revealed that miR-27a-3p overexpression profoundly promoted proliferation and migration in BT549 and 231 cell lines. Conversely, miR-27a-3p knockdown inhibited the proliferation and migration of 231 and BT549 cell lines. In addition, our data suggested that higher miR-27a-3p expression associated with shorter overall survival in patients with TNBC. Similarly, the relative expression of miR-27a-3p was higher in colon cancer patients compared to that in control ones.²⁷ Thus, it is necessary to discover the target involved in the oncogenic role of miR-27a-3p in TNBC.

In the present study, we predicted the candidate target genes for miR-27a-3p using miRWalk, TargetScan, PITA, and RNA22 platform. A given miRNA can target numerous genes. The tumor suppressor GSK3β tremendously attracted our attention. Further investigation found that miR-27a-3p inversely regulated the expression of GSK3β protein in TNBC cells. Luciferase reporter assay demonstrated that miR-27a-3p directly regulated GSK3β expression via binding to 3'UTR. GSK3β functions as a tumor suppressor via regulating cell proliferation and cell cycle progression in colorectal cancer.^{28,29} Ectopic expression of GSK3β suppresses lung cancer cell proliferation, migration, and invasion.³⁰ The absence of GSK3β breaks genomic stability to promote tumorigenesis and tumor progression.³⁰ A previous study also showed that miR-27a-3p regulates

proliferation and apoptosis of colon cancer cells by potentially targeting BTG1.²⁷ Currently, the biological role of GSK3β in TNBC is still unexplored. In our study, we demonstrated that GSK3β functioned as a novel tumor-suppressor gene in TNBC cells. More importantly, GSK3β silencing partially abolished miR-27a-3p knockdown induced inhibitory effects on TNBC cell growth. Altogether, our work suggested that miR-27a-3p contributed to TNBC progression possibly by attenuating GSK3β.

The Wnt/β-catenin signaling pathway is involved in cancer development.³¹ The Wnt/β-catenin signaling pathway activation has been shown to promote cell proliferation, invasion, and metastasis in nasopharyngeal carcinoma.^{32,33} The activation has also been shown to contribute to the maintenance of cancer stem cells in hepatocellular carcinoma.^{34,35} Our results also showed that GSK3β suppression by miR-27a-3p triggered the Wnt/β-catenin signaling pathway leading to TNBC proliferation and migration, and its downstream mechanisms need further investigation.

Conclusion

We found that miR-27a-3p expression was elevated in TNBC cell lines and higher miR-27a-3p expression associated with prognosis in patients with TNBC. Furthermore, our research shows that miR-27a-3p promoted cell proliferation and migration by inhibiting expression of GSK3β and activating Wnt/β-catenin signaling pathway. These observations suggested that miR-27a-3p may be a potential target of TNBC treatment.

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Disclosure

The authors declare that they have no competing interests.

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