

MiR-193 promotes cell proliferation and invasion by ING5/PI3K/AKT pathway of triple-negative breast cancer

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Abstract. – **OBJECTIVE:** Triple-negative breast cancers (TNBC) are a subtype of breast cancer lacking of estrogen receptor (ER), progesterone receptor (PR), and human EGF-like receptor 2 (HER2). MiR-193 always acted as an oncogene and promoted toxic aldehyde accumulation and tyrosine hydroxylase dysfunction. The purpose of this study is to explore the function of miR-193 in triple-negative breast cancer.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the mRNA level of miR-193 expression in 50 cases of TNBC tissues and para-cancerous specimens. Also, the relation between miR-193 level and the overall survival of TNBC patient was analyzed. MiR-193 mimic and miR-193 inhibitor oligos, as well as the corresponding negative control, were synthesized from RiboBio (Guangzhou, China).

RESULTS: MiR-193 expression was higher in triple-negative breast cancer tissues and cell lines than the corresponding adjacent non-tumor tissues and normal cell lines. Upregulation of miR-193 predicted poor prognosis of TNBC patients. Overexpression of miR-193 promoted cell proliferation and invasion, while that was suppressed by the knockdown of miR-193. MiR-193 binds to the 3'-UTR of an inhibitor of growth family member 5 (ING5) mRNA to mediate the expression of ING5 in TNBC cells. The knockdown of miR-193 inhibited cell invasion-mediated epithelial-mesenchymal transition (EMT). Furthermore, the knockdown of miR-193 suppressed cell proliferation through the ING5/phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signal pathway.

CONCLUSIONS: MiR-193 enhanced cell invasion-mediated EMT and improved cell proliferation through the ING5/PI3K/AKT signal pathway in triple-negative breast cancer. The newly identified miR-193/ING5/PI3K/AKT axis provides novel insight into the pathogenesis of triple-negative breast cancer.

Key Words:

MiR-193, Triple negative breast cancer, ING5, Invasion.

Introduction

Breast cancer (BC) is one of the most common female cancers, ranking first in malignant neoplasm among women in China¹. Triple-negative breast cancer (TNBC) was an invasive subtype of breast cancer lacking the expression of three hormone receptors, i.e., estrogen receptor (ER), progesterone receptor (PR), and human EGF-like receptor 2 (HER2)^{2,3}. It is urgent to develop therapeutic targets and prognostic markers for the treatment of triple-negative breast cancer.

MicroRNAs (miRNAs), a class of 19-25 nucleotides non-coding RNAs, lead to the mRNA degradation or the inhibition of protein translation to mediate gene expression at a post-transcriptional level⁴. Dysregulation of several miRNAs may play crucial biological processes in promoting tumorigenesis in triple-negative breast cancer, including miR-613, miR-770, miR-221, and miR-193⁵⁻⁸. MiR-193 was abnormally expressed in many diseases⁹⁻¹¹, including telocyte, pulmonary hypertension, and diabetic cardiomyopathy. It has been demonstrated¹² that miR-193 promoted toxic aldehyde accumulation and tyrosine hydroxylase dysfunction in cerebral ischemia. Accordingly, miR-193 was involved in embryo-implantation in mouse uterus¹³. Therefore, we strongly believe that miR-193 may play crucial roles in triple-negative breast cancer.

Inhibitor of growth family member 5 (ING5), which contains a PHD-type zinc finger, interacts with the tumor suppressor p53 and p300, suggesting its role in transcriptional regulation¹⁴. ING5 has been reported to be a member of ING gene family, which acted as tumor suppressors and were associated with cell apoptosis and repair of deoxyribonucleic acid (DNA) damage^{15,16}. ING5 may play an important role as a potential target for the tumorigenesis and therapy of os-

teosarcoma, ovarian, and glioma¹⁷⁻¹⁹. In bladder cancer, Li et al²⁰ revealed that ING5 increased the chemoresistance and inhibited the DNA damage response pathway. Moreover, ING5 has been reported to suppress the proliferation and invasion in esophageal squamous cell carcinoma²¹. Even in breast cancer, ING5 was downregulated and inhibited the epithelial-mesenchymal transition (EMT) by suppressing the PI3K/AKT pathway²². Therefore, we strongly believe that miR-193 may regulate the progress of triple-negative breast cancer through ING5.

Patients and Methods

Patients and Tissue Samples

In the Affiliated Hospital of Qingdao University, 50 patients with triple-negative breast cancer were selected from January 2015 to December 2017. 50 pairs of cancer tissues and corresponding adjacent tissues were obtained. The samples were instantly frozen in liquid nitrogen and stored in a -80°C freezer. All the patients did not receive radiotherapy or chemotherapy before the operation. All patients provided written informed consent. Approval for the investigation was received from the Ethics Committee of the Affiliated Hospital of Qingdao University.

Cell Culture and Preparation

Two TNBC cell lines MDA-MB-231 and BT483, as well as one normal epithelial cell line MCF-10A, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) was employed to culture the MDA-MB-231 and BT483 cells, while MCF-10A cells were cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12; Hyclone, South Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO_2 . Both the mediums were contained with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA).

Vectors and Transfection

The miR-193 mimic and the miR-193 inhibitor oligonucleotides, as well as the corresponding negative control, were synthesized from Ribobio (Guangzhou, China). MDA-MB-231 cells were seeded in 6-well plate and cultured overnight for transfection. In accordance with the manufacturer's instructions, Lipofectamine

2000 (Invitrogen, Carlsbad, CA, USA) and equal amounts of oligo fragments were diluted by Opti-MEM/Reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Once the two solutions were mixed, the mixture was added into a 6-well plate. For the cells stably expressing the miR-193 inhibitor, the oligo fragment was inserted in the vector and added into cells. Then, cells were selected by Geneticin (G418; Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The reverse transcription of miR-193 was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The TaqMan MicroRNA assay was conducted to undertake the qRT-PCR.

The first complementary deoxyribonucleic acid (cDNA) strand of ING5 was synthesized using the Reverse Transcription System (Thermo Fisher Scientific, Waltham, MA, USA). The PCR reaction was carried out by using the Power SYBR Green PCR Master Mix (Ambion/Applied Biosystems, Foster City, CA, USA) on an ABI 7300HT system (Applied Biosystems, Foster City, CA, USA). The mRNA levels of miR-193 and ING5 were normalized to U6 snRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for PCR were as follows: miR-193 forward 5'-AACTGG-CCTACAAAGTC-3' and reverse 5'-GTGCAG-GGTCCGAGGT-3'; U6 forward 5'-GCTTCG-GCAGCACATATACTAAAAT-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; ING5 forward 5'-ACCAGAGGACGGAAGATA-AG-3' and reverse: 5'-TGCACTTGCTGTAGGC-GTTC-3'; GAPDH forward 5'-ACAACCTTTGG-TATCGTGGAAGG-3' and reverse 5'-GCCAT-CACGCCACAGTTTC-3'.

Western Blot

MDA-MB-231 cells were lysed by the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), containing 1% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) and utilized to inhibit the degradation of proteins. The bicinchoninic acid (BCA) Protein Quantification Kit (Solarbio, Beijing, China) was conducted to measure the concentration of total proteins. Once separated through 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by elec-

trophoresis, the proteins were then transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% skimmed milk for 1 h at room temperature and incubated using primary antibodies, which were ING5 (1:1,000; Protein-tech, Chicago, IL, USA), E-cadherin (1:1000; Abcam, Cambridge, MA, USA), N-cadherin (1:1000; Abcam, Cambridge, MA, USA), Vimentin (1:1000; Abcam, Cambridge, MA, USA) p-PI3K (1:1000, Cell Signaling, San Jose, CA, USA), PI3K (1:1000, Cell Signaling, San Jose, CA, USA), p-AKT (1:1000, Cell Signaling, San Jose, CA, USA), AKT (1:1000, Cell Signaling, San Jose, CA, USA), and GAPDH (1:6,000 dilution; Sigma-Aldrich, St Louis, MO, USA). Later, the anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated secondary antibody were conducted to incubate the membranes, and the signal was measured by enhanced chemiluminescence (ECL; Pharmacia Biotech, Arlington, MA, USA).

Proliferation Assay

The cell proliferative ability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which was using MTT (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA) solutions. Before transfection, a density of 5×10^3 cells/well of MDA-MB-231 cells were seeded into 96-well plates and cultured at 37°C. After cultured 24, 48, 72, and 96 h, added 10 μ L MTT into each well and incubated for 4 h at 37°C. The medium contained with MTT was removed and 150 μ L DMSO added into each well, and then the absorbance of cell proliferation was measured at 490 nm.

Transwell Assay

The invasive cell ability was calculated by transwell chamber (8 μ m; Biosciences, San Jose, CA, USA) covered with Matrigel (Clontech; Mountain View, CA, USA). The transwell chamber was put into 24-well plate and seeded 100 μ L cell suspension into the upper chamber, which was suspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Hyclone; South Logan, UT, USA) without serum. Meanwhile, 600 μ L RPMI-1640 medium with 15% FBS was placed into the lower chamber. After incubated 48 h at 37°C in 5% CO₂, the cells still at the upper surface were removed using a cotton swab. Next, cells which moved to the lower surface were fixed and stained using methanol

and 0.1% crystal violet. The cell invasion ability is represented by the number of cells that pass through the membrane.

Luciferase Reporter Assays

TargetScan predicted the target genes of miR-193. ING5 was predicted to be a target gene of miR-193. To clarify miR-193 directly binding to ING5, the binding sequences were mutated from GGCCAGU to CCGGUCA. The 3'-UTR fragment of ING5 mRNA containing the wild type or mutated sequence was inserted into the pmirGlo vector, which was designated as pmirGlo-ING5-WT (WT) and pmirGlo-ING5-MUT (MUT), respectively. According to the manufacturer's recommendations, the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized to perform the transfection in MDA-MB-231 cells. 48 h post-transfection, the firefly luciferase activity was calculated using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) with *Renilla* luciferase as normalization.

MiR-193 Promotes TNBC Xenograft Growth

The nude mice with 4 weeks old were purchased from Charles River Laboratories (Beijing, China). After one week of incubation to acclimate the environment, MDA-MB-231 cells stably expressing miR-193 inhibitor were subcutaneously inoculated in nude mice to illuminate the role of miR-193 on the growth of TNBC cells *in vivo*. Tumor volumes were evaluated every 3 days and the mice were sacrificed and went out of the xenograft after cell implantation for 26 days. All animal experiments were performed in the Animal Laboratory Center of the Affiliated Hospital of Qingdao University and approved by the Affiliated Hospital of Qingdao University Animal Care and Use Committee.

Statistical Analysis

All the data are presented as means \pm SD (standard deviation) and the statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 16.0 software (Chicago, IL, USA). The student's *t*-test was performed to evaluate the significant difference between the two groups. Comparison between multiple groups was made using the One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ was statistically significant.

Results

MiR-193 was Upregulated in TNBC Tissues, and Upregulation of MiR-193 Predicted Poor Prognosis

The expression of miR-193 was calculated in TNBC and the corresponding adjacent non-tumor tissues by qRT-PCR. The expression of miR-193 was higher in triple-negative breast cancer tissues than in the corresponding non-tumor tissues ($p < 0.0001$) (Figure 1A). The overall survival was evaluated by the Kaplan-Meier method. We found that the overexpression of miR-193 predicted poor prognosis of triple-negative breast cancer patients ($p = 0.034$) (Figure 1B).

MiR-193 Promoted the Proliferation and Invasion in TNBC

The mRNA level of miR-193 was evaluated in cell lines. We discovered that the expression of miR-193 was higher in MCF-10A than TNBC cells MDA-MB-231 and BT-483 ($p = 0.003$ and 0.0011) (Figure 2A). To explore the function of miR-193 in TNBC, the miR-193 mimic and the miR-193 inhibitor were conducted to overexpress ($p < 0.0001$) and knockdown of ($p = 0.0023$) miR-193 in MDA-MB-231 cells (Figure 2B). MTT assay was utilized to evaluate the proliferative ability after transfecting the miR-193 mimic or the miR-193 inhibitor. The miR-193 mimic promoted ($p = 0.030$, 0.010 and 0.0006) the proliferation, while the miR-193 inhibitor suppressed ($p = 0.015$ and 0.0035) the proliferation in MDA-MB-231

cells (Figure 2C). Moreover, the invasive ability was calculated by transwell assay after exogenous alteration of the miR-193 expression. The same result with proliferation, the invasive ability was increased ($p = 0.013$) by the miR-193 mimic, whereas it was reduced ($p = 0.018$) by the miR-193 inhibitor (Figure 2D).

ING5 Was a Target Gene of MiR-193 in TNBC Cells

TargetScan was conducted to predict the target genes of miR-193. ING5 was discovered to be a potential target gene of miR-193. To verify whether miR-193 bound directly to ING5, the binding sequences on the 3'-UTR of ING5 mRNA were mutated from GGCCAGU to CCGGUCA (Figure 3A). The wild type and the mutant 3'-UTR fragments were inserted into a pmirGlo luciferase vector, named as pmirGlo-ING5-WT (WT) and pmirGlo-ING5-MUT (MUT), respectively. Then, MDA-MB-231 cells co-transfected the miR-193 mimic and WT or MUT and calculated the luciferase activities. Compared to the control, the miR-193 mimic reduced ($p = 0.0013$) the luciferase activity of wild type 3'-UTR of ING5 mRNA, whereas exhibited no inhibitory effects on the luciferase activity of mutant ING5 3'-UTR in MDA-MB-231 cells ($p = 0.9637$) (Figure 3B). In addition, the mRNA level of ING5 was reduced ($p = 0.0022$) by the miR-193 mimic, while it was increased by the miR-193 inhibitor in MDA-MB-231 cells ($p = 0.0004$) (Figure 3C).

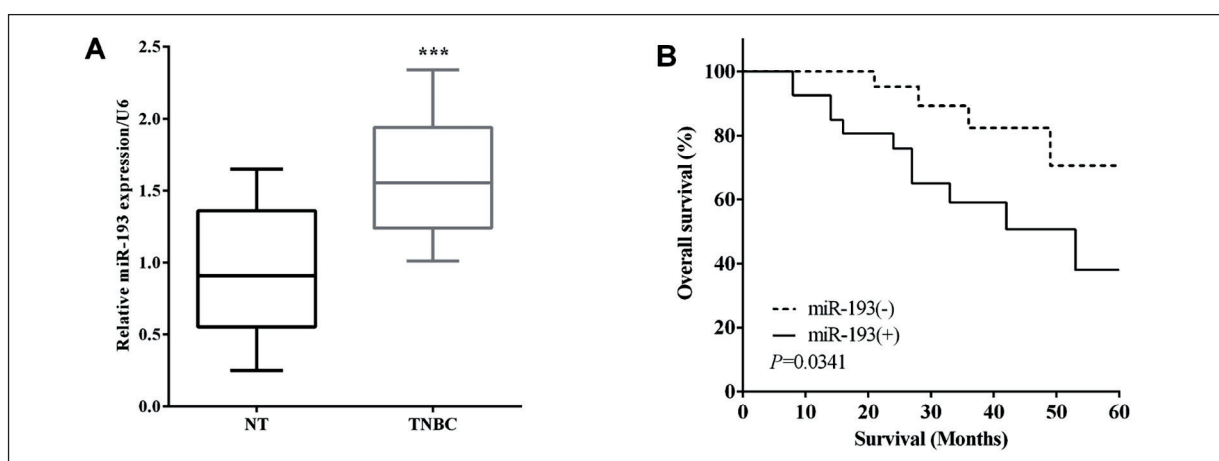


Figure 1. MiR-193 was upregulated in TNBC tissues and upregulation of miR-193 predicted poor prognosis. **A**, Expression of miR-193 was lower in triple negative breast cancer tissues than the corresponding non-tumor tissues. **B**, Kaplan-Meier revealed that overexpression of miR-193 predicted poor prognosis.

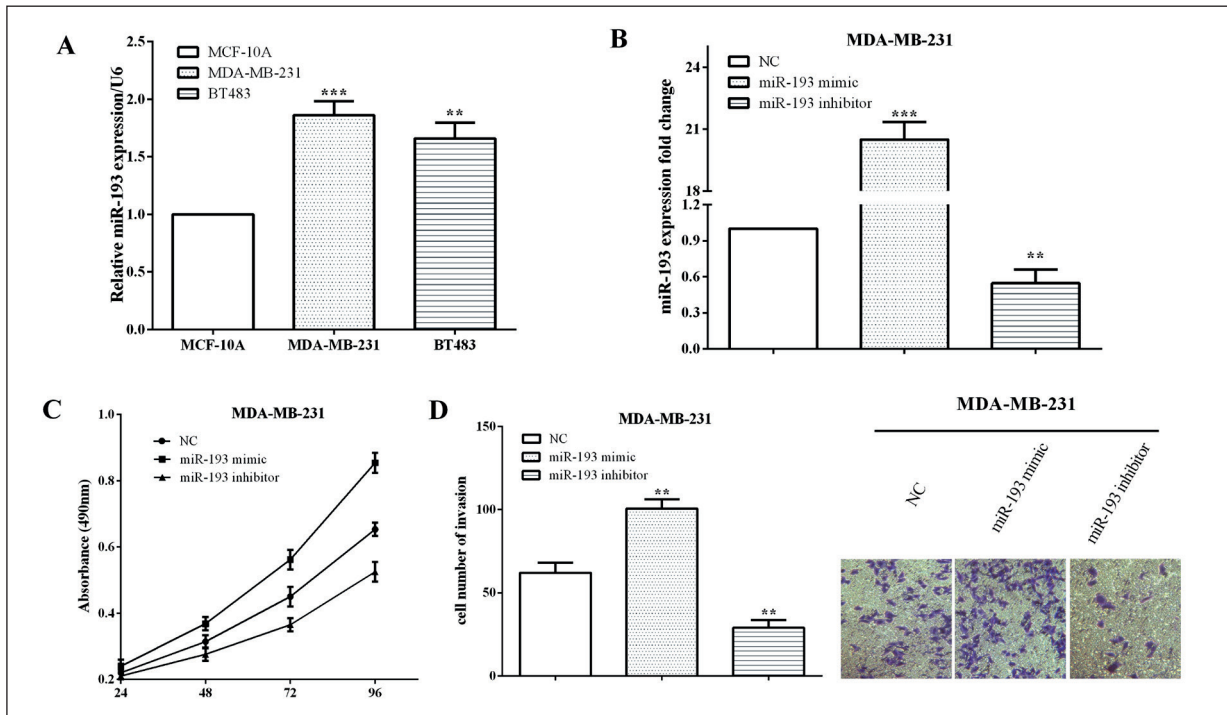


Figure 2. MiR-193 inhibited the proliferation and invasion in TNBC **A**, Expression of miR-193 was lower in MCF-10A cells than TNBC cell lines MDA-MB-231 and BT-483. **B**, MiR-193 mimic and the miR-193 inhibitor were conducted to overexpress and knockdown of miR-193 in MDA-MB-231 cells. **C**, MiR-193 mimic promoted the proliferation while the miR-193 inhibitor suppressed the proliferation in MDA-MB-231 cells. **D**, Transwell assay indicated the invasive ability was increased by the miR-193 mimic, whereas it was reduced by the miR-193 inhibitor (magnification: 200 \times).

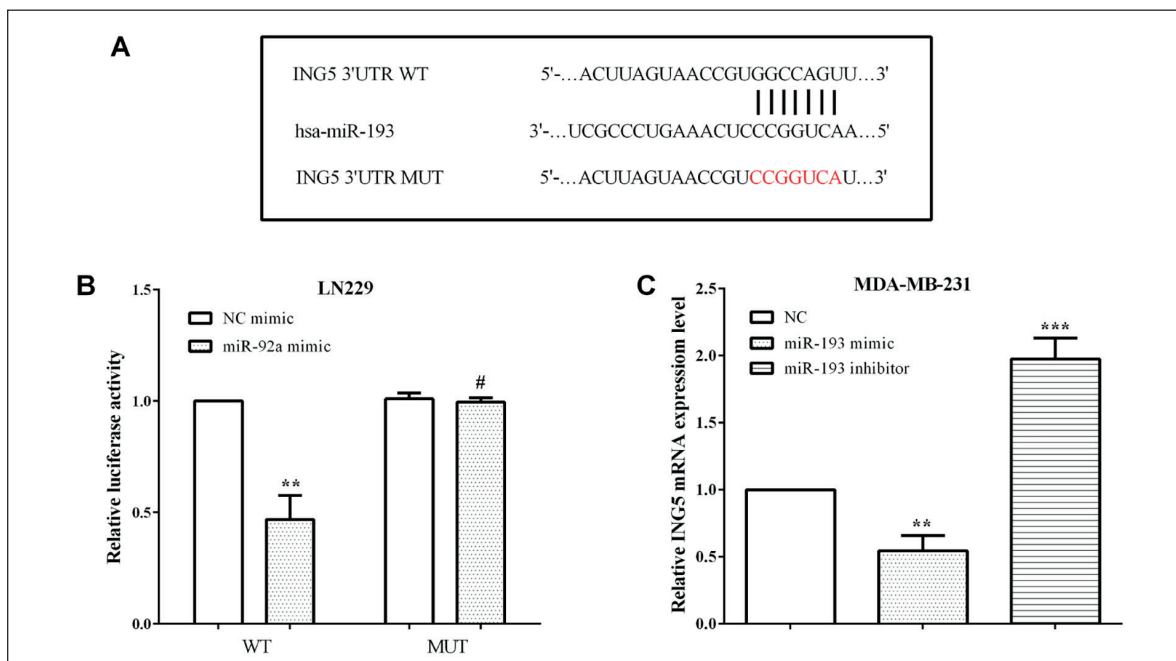


Figure 3. ING5 was a direct target gene of miR-193 in TNBC cells **A**, ING5 was discovered to be a potential target of miR-193 by TargetScan. **B**, MiR-193 mimic reduced the luciferase activity of wild type ING5 3'-UTR, whereas exhibited no inhibitory effects on the luciferase activity of mutant ING5 3'-UTR in MDA-MB-231 cells. **C**, mRNA level of ING5 was reduced by the miR-193 mimic, while it was increased by the miR-193 inhibitor in MDA-MB-231 cells.

MiR-193 Promoted TNBC the Proliferation and Invasion via PI3K/AKT Signal Pathway

The expression of ING5 was calculated in tissues. We discovered that ING5 was downregulated in TNBC tissues compared with the corresponding non-tumor tissues ($p < 0.0001$) (Figure 4A). Moreover, the expression of ING5 was also assessed to be higher in MCF-10A than MDA-MB-231 ($p = 0.0006$) and BT-483 ($p = 0.0060$) (Figure 4B). To explore the mechanism of miR-193 on TNBC the proliferation and invasion, Western blot was performed to evaluate the protein levels of EMT markers and the PI3K pathway. As expected, the knockdown of miR-193 improved the protein levels of ING5 and E-cadherin, whereas it reduced the expression of N-cadherin and Vimentin in MDA-MB-231 cells (Figure 4C). Moreover, the expression of p-PI3K and p-AKT was reduced by miR-193 inhibitor in MDA-MB-231 cells (Figure 4D). Taken together, the knockdown of miR-193 suppressed the EMT and PI3K/AKT signal pathway in MDA-MB-231 cells.

MiR-193 Promoted the Xenograft Growth In Vivo

The cells used for xenograft experiments were MDA-MB-231 cells. They stably transfected the miR-193 inhibitor or control plasmid and subcutaneously injected into the nude mice. The xenograft tumors volume was calculated every 3 days. MiR-193 knockdown group had a slower growth rate than control group (Figure 5A). Further results indicated that knockdown of miR-193 inhibited the growth of TNBC xenograft ($p = 0.0020$) (Figure 5B).

Discussion

Triple-negative breast cancer was a subtype of breast carcinoma lacking estrogen receptor (ER), progesterone receptor (PR), and human EGF-like receptor 2 (HER2)^{2,3}. To develop therapeutic targets and prognostic markers for the treatment of triple-negative, breast cancer is necessary.

MiRNAs were non-coding RNAs, which could mediate the expression of the target gene at

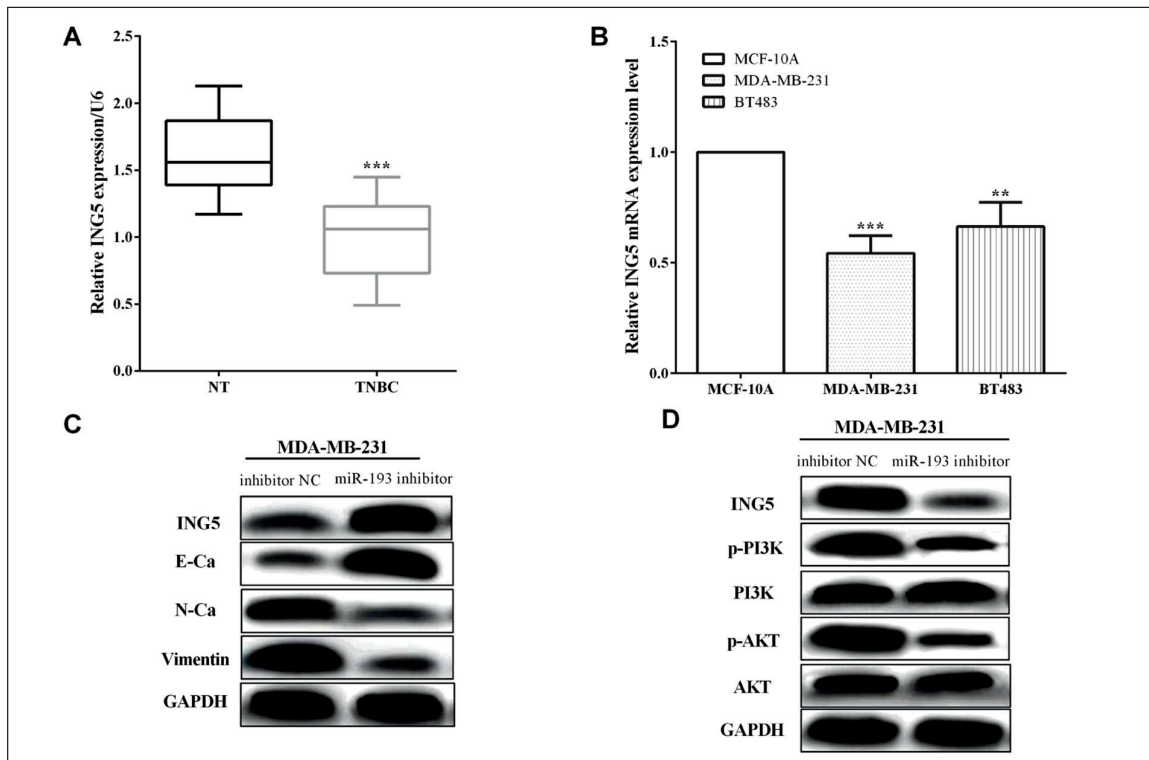


Figure 4. MiR-193 suppressed the proliferation and invasion through PI3K/AKT signal pathway in TNBC cells. **A**, ING5 was downregulated in TNBC tissues versus the corresponding non-tumor tissues. **B**, Expression of ING5 was assessed higher in MCF-10A than MDA-MB-231 and BT-483. **C**, Knockdown of miR-193 suppressed the EMT in MDA-MB-231 cells **D**, Knockdown of miR-193 inhibited PI3K/AKT signal pathway in MDA-MB-231 cells.

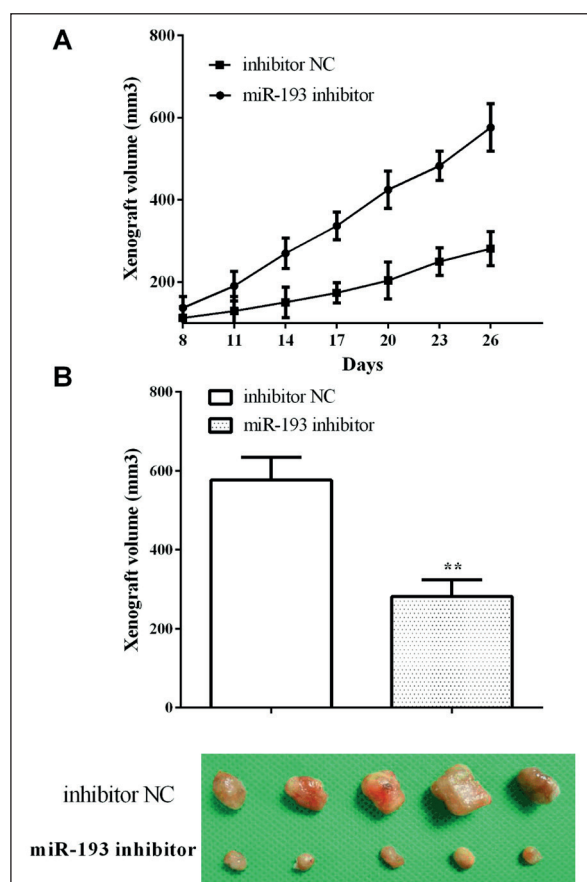


Figure 5. MiR-193 promoted the growth of TNBC xenograft *in vivo*. **A**, Knockdown of miR-193 inhibited the growth of TNBC xenograft. **B**, It had a smaller tumor volume of cells knockdown of miR-193 than the control group.

post-transcriptional levels⁴. MiR-193 could mediate angiogenesis and promoted toxic aldehyde accumulation and tyrosine hydroxylase dysfunction in disease, diabetic cardiomyopathy, and cerebral ischemia. Liu et al²³ indicated that miR-193 suppressed the growth and migration in renal cell carcinoma cells. Similarly, Jian et al²⁴ discovered that miR-193 inhibited tumor proliferation, migration, and chemoresistance in gastric cancer cells. In addition, miR-193 has been reported to be upregulated in breast cancer and promoted the growth, survival, and motility of SKBR3 cell⁸. Our results were consistent with all the findings, miR-193 was found to be upregulated in triple-negative breast cancer tissues, and a high level of miR-193 predicted a shorter overall survival rate. In MDA-MB-231 cells, the overexpression of miR-193 promoted the proliferation and invasion, whereas those were inhibited by the

knockdown of miR-193. Moreover, knockdown of miR-193 suppressed the growth of TNBC cell xenograft tumor.

ING5, has been reported to be a tumor suppressor, was connected with the repair of DNA damage, and it acted as a potential target for tumorigenesis and therapy^{15,17-19}. It was revealed that ING5 suppressed the proliferation and invasion through the regulation of the Akt/NF- κ B/MMP-9 signaling pathway in esophageal squamous cell carcinoma cells²¹. Also, Gao et al²⁵ indicated that ING5 overexpression inhibited the proliferation, invasion, and EMT in thyroid cancer. We proposed consistent results that ING5 was downregulated in triple-negative breast cancer tissues and cell lines. We verified that ING5 was a direct target gene of miR-193, which was consistent with the findings of Roy et al²⁶. Moreover, the knockdown of miR-193 mediated the invasion by inhibiting EMT in TNBC cells. The knockdown of miR-193 suppressed the proliferation through the ING5/PI3K/Akt signal pathway in MDA-MB-231 cells. This was the first time to propose the connection between miR-193 and ING5 in TNBC.

Conclusions

We indicated that the expression of miR-193 was higher in TNBC tissues than the corresponding adjacent non-tumor tissues. Upregulation of miR-193 was associated with poor prognosis of TNBC patients. The proliferative and invasive abilities were promoted by the overexpression of miR-193, while those were suppressed by the knockdown of miR-193. MiR-193 directly binds to the 3'-UTR of ING5 mRNA to mediate the expression of ING5 in TNBC cells. Knockdown of miR-193 inhibited the invasion-mediated EMT in MDA-MB-231 cells and suppressed the proliferation through the ING5/PI3K/AKT signal pathway in MDA-MB-231 cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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