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Overexpression of miR-3196 suppresses cell proliferation and induces cell apoptosis through targeting ERBB3 in breast cancer

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Abstract. – OBJECTIVE: Breast cancer is one of the most common malignancies worldwide. MicroRNAs (MiRNAs) have been identified to influence cell behaviors through epigenetic post-transcriptional gene regulation. Therefore, the aim of this study was to determine the role of miR-3196 in the proliferation and apoptosis of breast cancer.

MATERIALS AND METHODS: Human breast cancer cell lines (MCF-7 and MDA-MB-231) were obtained and cultured. The expression level of miR-3196 in breast cancer tissues was detected using Real Time-Polymerase Chain Reaction (RT-PCR). The effects of miR-3196 on the proliferation and apoptosis of breast cancer cells were analyzed by cell counting kit-8 (CCK-8) assay and TUNEL assay, respectively. In addition, the interaction between miR-3196 expression and erb-b2 receptor tyrosine kinase 3 (ERBB3) expression, as well as the mechanism of miR-3196 regulating ERBB3 in breast cancer, were also addressed by RT-PCR, Western blot, and luciferase reporter gene assay.

RESULTS: MiR-3196 was lowly expressed in breast cancer tissues. Overexpression of miR-3196 could repress the proliferation and induce the apoptosis of breast cancer cells via targeting the 3'UTR of ERBB3.

CONCLUSIONS: Our findings provide novel insights into the role of miR-3196 in breast cell proliferation and apoptosis. Meanwhile, this study suggests that miR-3196 can serve as a potential biomarker and therapeutic target for breast cancer.

Key Words:
MiR-3196, ERBB3, Proliferation, Apoptosis, Breast cancer.

Introduction

In the world, breast cancer is one of the most common malignant tumors in women with high morbidity and mortality. Many advances have been progressed in the diagnosis and treatment of breast cancer, including surgery, radiotherapy, chemotherapy and hormone therapy. However, the survival of breast cancer patients remains poor[1]. Thus, exploration of the underlying mechanism of breast cancer progression is of great importance for therapeutic strategies of breast cancer. MicroRNAs (miRNAs) are a class of small, non-coding RNAs with 19 to 25 nucleotides in length. They modulate the silencing of mRNA transcripts *via* annealing to 3'-UTR. Meanwhile, miRNAs also repress translation or initiate the degradation of transcript, thereby promoting different gene regulation[2-4]. Increasing evidence has demonstrated that the dysregulation of miRNAs is related to multiple biological processes. For example, miR-661 can enhance tumor invasion and metastasis *via* repressing retinoblastoma 1 (RB1) in non-small cell lung cancer[5]. MiR-140 suppresses the proliferation, migration and invasion of gastric cancer cells *via* regulating YES1[6]. In breast cancer, inhibition of pyruvate dehydrogenase complex, component X (PDHX) by miRNA-27b deregulates cell metabolism and enhances cell growth[7]. However, the exact role of miR-3196 in breast cancer needs to be further explored. In current study, we found that miR-3196 maintained a low expression level in breast cancer tissues. Besides, overexpres-

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sion of miR-3196 could suppress the proliferation and induce the apoptosis of breast cancer cells. Mechanistically, miR-3196 negatively regulated erb-b2 receptor tyrosine kinase 3 (ERBB3) gene by binding to its 3'-UTR. Overall, our findings demonstrated a novel factor in the regulation of cell proliferation and apoptosis. Furthermore, miR-3196 might be used as an important biomarker for the diagnosis and targeted therapy of breast cancer.

Materials and Methods

Cell Culture

Human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin in a 37°C, 5% CO_2 incubator.

Cell Transfection

One day before transfection, cells were seeded into 6-well plates at a density of 1.5×10^5 cells / mL. MCF-7 and MDA-MB-231 cells were transfected with miR-3196 mimics (a final concentration of 5 μM) and relative negative control (NC) (GenePharma, Shanghai, China) according to the manufacturer's instructions of Lipofectamine RNAiMAX Reagent (Life Technologies, Grand Island, NY, USA).

RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

24 hours after cell transfection, total RNA was extracted from cells in strict accordance with the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). The concentration of extracted RNA was detected by NanoDrop 2000. RNA (500 ng) was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative expression level of ERBB3 was detected by qRT-PCR Primer Assays and SYBR Green Master Mix (Qiagen, Valencia, CA, USA) in an ABI 7900HT System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Primers used in this study were as follows: ERBB3, F: 5'- CAAGATTCCAGTCTGCATTAAAGTC-3', R: 5'-CAGCATATGATCTGTCACAGCTTG-3'; miR-3196, F: 5'-CCTGTGTATGCATCCTCGACTG-3', R: 5'-CTGGCGTGTAATGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

Western blot was conducted to determine the protein expression levels of ERBB3, Cyclin D1, Ki67, Bcl-2 and Caspase-3 as previously described[8]. Radio-immunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was used for total protein extraction in cells after washing with phosphate-buffered saline (PBS). Extracted protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then immersed in 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) containing specific primary antibodies at 4°C overnight. After washing with Tris-Buffered Saline and Tween 20 (TBST) buffer for 3 times (10 min for each time), the membranes were incubated with horseradish peroxide (HRP) conjugated secondary antibody (Abnova, Taiwan) at room temperature for 2 hours. Finally, immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates at a density of 1×10^3 cells per well, followed by culture in DMEM medium (100 μL) supplemented with 1% FBS. After culture for 24, 48 and 72 hours, 10 μL CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) were added in each well, followed by incubation for 2 h in dark. The absorbance at the wavelength of 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

TUNEL Staining

According to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique, cells were stained with *in situ* cell death detection kit. Briefly,

cultured cell smears were first fixed with 4% paraformaldehyde in PBS, and then permeabilized with 0.1% sodium citrate containing 0.1% Triton X-100 on ice. Subsequently, the cells were incubated with the TUNEL reaction mixture. After rinsing with PBS, the cells were stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich, St. Louis, MO, USA) and visualized under a fluorescence microscope. The number of TUNEL-positive nuclei (green fluorescence) was expressed as the percentage of total nuclei (blue fluorescence).

Luciferase Reporter Gene Assay

ERBB3 3'-UTR with wild-type or mutant miR-3196 sequence was inserted into the pGL3-reporter plasmid (Ambion, Thermo Fisher Scientific, Waltham, MA, USA), respectively. MCF-7 cells were first seeded into 24-well plates. Subsequently, the cells were transfected with a mixture of 0.2 μg pGL3-ERBB3 or mutant, 0.1 μg β-galactosidase (β-gal) expression plasmid (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and equal amount (20 pmol) of RNA. The β-gal plasmid was used as a control. Luciferase activity was detected using a luciferase assay kit (Promega, Madison, WI, USA) after 24 hours.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software was used for all statistical analysis. Experimental data were expressed as mean ± SD. Student's *t*-test was used to test the differences between the experimental group and the control group. Two-tailed tests were used, and $p < 0.05$ was considered statistically significant.

Results

MiR-3196 Was Lowly Expressed in Breast Cancer Tissues

We first performed RT-PCR to detect the expression level of miR-3196 in breast cancer. As shown in Figure 1A, the expression of miR-3196 in breast cancer tissues was significantly lower than that of adjacent normal tissues. These results indicated that dysregulation of miR-3196 might be involved in the progression of breast cancer. To further detect the underlying function of miR-3196 in breast cancer progression, we transfected miR-3196 mimics into breast cancer cells to upregulate its expression. Transfection efficiency was confirmed by RT-PCR (Figure 1B, 1C).

Overexpression of miR-3196 Repressed the Proliferation of Breast Cancer Cells

Dysregulation of cell proliferation is an important factor in tumor progression. To determine whether overexpression of miR-3196 could influence cell behavior, CCK-8 assay was performed to detect cell proliferation. Results showed that overexpression of miR-3196 significantly repressed cell proliferation (Figure 2A, 2B). The expression levels of multiple genes involved in cell proliferation were significantly altered, such as Cyclin D1 and Ki67. Subsequently, we used Western blot analysis to detect the protein expression levels of Cyclin D1 and Ki67. Data showed that Cyclin D1 and Ki67 were both remarkably reduced in breast cancer cells transfected with miR-3196 mimics (Figure 2C, 2D). Overall, these findings demonstrated that overexpression of miR-3196 could repress breast cancer cell proliferation.

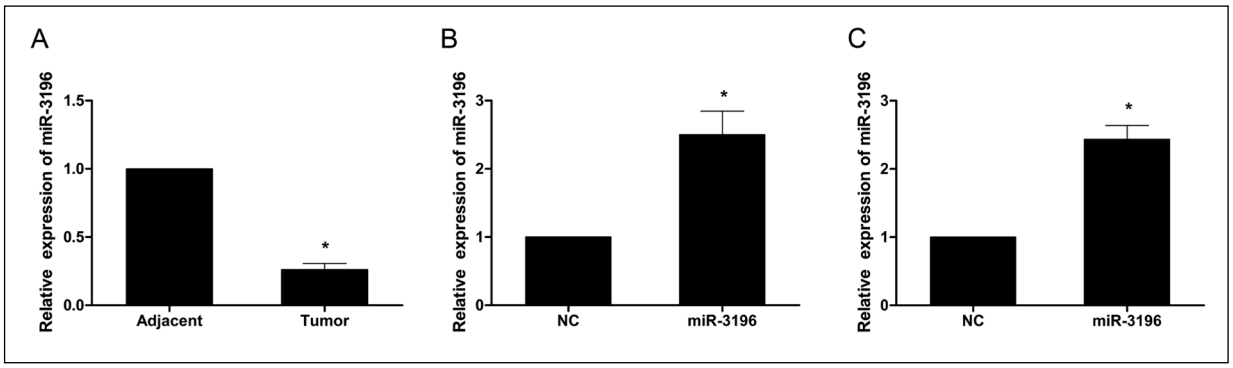

Figure 1. MiR-3196 was lowly expressed in breast cancer tissues. ***A,*** The expression levels of miR-3196 in breast cancer tissues and adjacent normal tissues were detected by RT-PCR. ***B,*** (MCF-7) and ***C,*** (MDA-MB-231) cells were transfected with miR-3196 mimics. Transfection efficiencies were confirmed by RT-PCR. *$p < 0.05$.

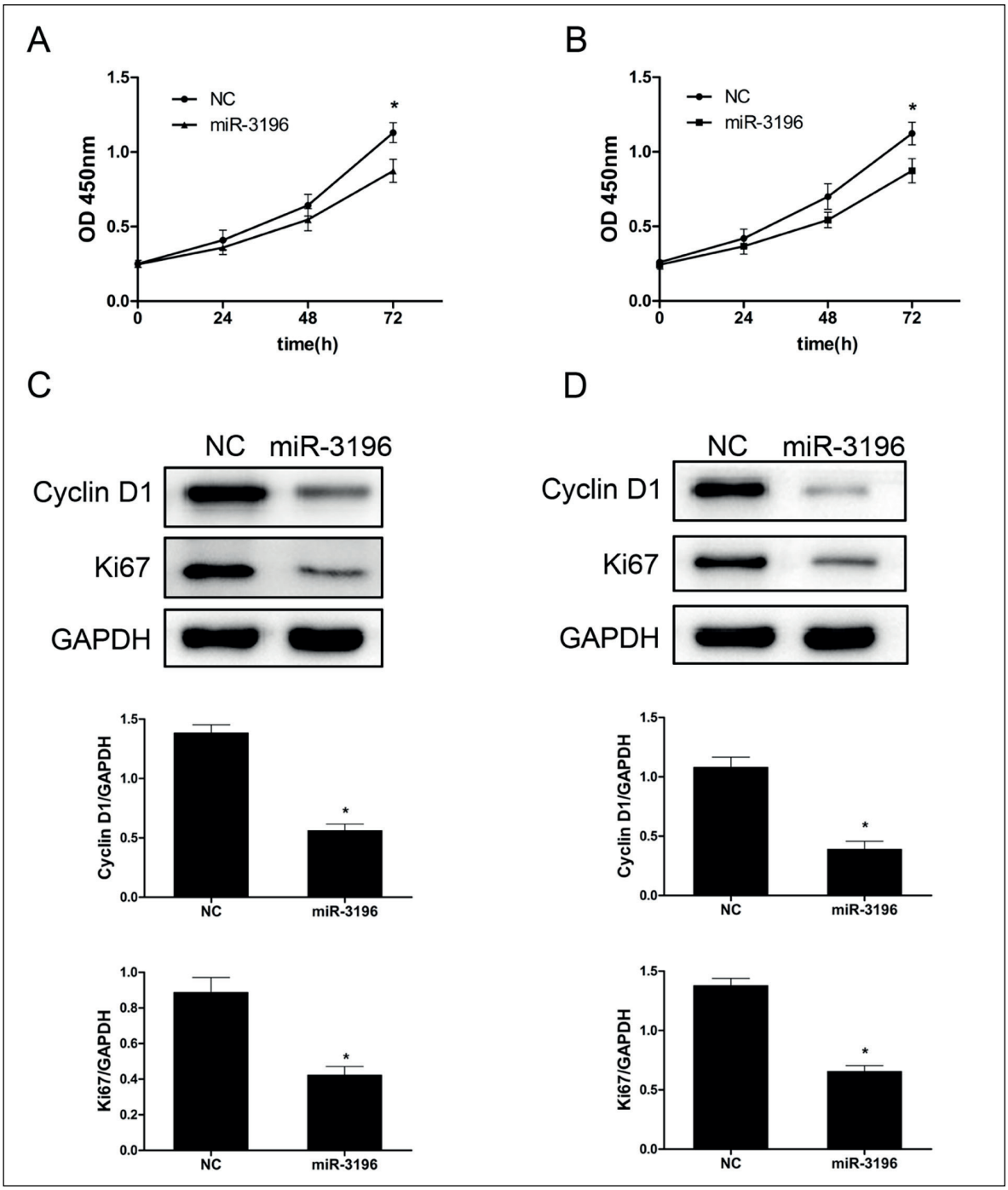

Figure 2. Overexpression of miR-3196 could repress breast cancer cell proliferation. CCK-8 assay was used to detect the proliferation of ***A,*** (MCF-7) and ***B,*** (MDA-MB-231) cells. The expression levels of Cyclin D1 and Ki67 in ***C,*** (MCF-7) and ***D,*** (MDA-MB-231) cells were detected by Western blot analysis. *$p < 0.05$.

Overexpression of miR-3196 Induced the Apoptosis of Breast Cancer Cells

Furthermore, cell apoptosis is also a crucial factor influencing tumor progression. Thus, to evaluate whether overexpression of miR-3196 affected cell apoptosis, we performed TUNEL assay. Results showed that overexpression of miR-3196 could significantly induce cell apoptosis when compared with the control group (Figure 3A, 3B). Meanwhile, the expression levels of mul-

tiple genes involved in cell apoptosis were significantly changed, including Bcl-2 and Caspase-3. Subsequently, we investigated the protein expression levels of Bcl-2 and Caspase-3 by western blot analysis. Results indicated that the protein expression of Bcl-2 in cells transfected with miR-3196 mimics was significantly reduced, whereas Caspase-3 expression was remarkably increased (Figure 3C, 3D). In summary, these findings illustrated that overexpression of miR-3196 could induce breast cancer cell apoptosis.

MiR-3196 Targeted the 3'-UTR of ERBB3

A growing number of studies have reported that miRNAs can influence the cellular behaviors of many diseases by inversely modulating target genes. In this study, miRNA target prediction software starBase (http://starbase.sysu.edu.cn/) was used to screen the potential target genes of miR-3196. Results demonstrated that ERBB3 was a potential target gene of miR-3196. Hence, we sought to determine the correlation between miR-3196 with ERBB3. ERBB3 was overexpressed and participated in the progression of breast cancer. The potential binding site predicted by starBase database was shown in Figure 4A. To validate this interaction, we further conducted a luciferase reporter gene assay. Results revealed that the relative luciferase activity in cells transfected with miR-3196 mimics and ERBB3 was significantly decreased when compared with that of the control group (Figure 4B). Inversely, the vector containing 3'-UTR mutant sequence was not modulated by miR-3196. In additional, RT-PCR and western blot assay also validated this negative regulation. As expected, both the mRNA and protein levels of ERBB3 were significantly reduced by upregulating miR-3196 (Figure 4C-4F). All the above data suggested that miR-3196 targeted the 3'-UTR of ERBB3.

Discussion

Breast cancer is a malignant tumor that occurs in the epithelial tissue of the breast gland. It has become a common malignant tumor seriously threatening women's physical and mental health.

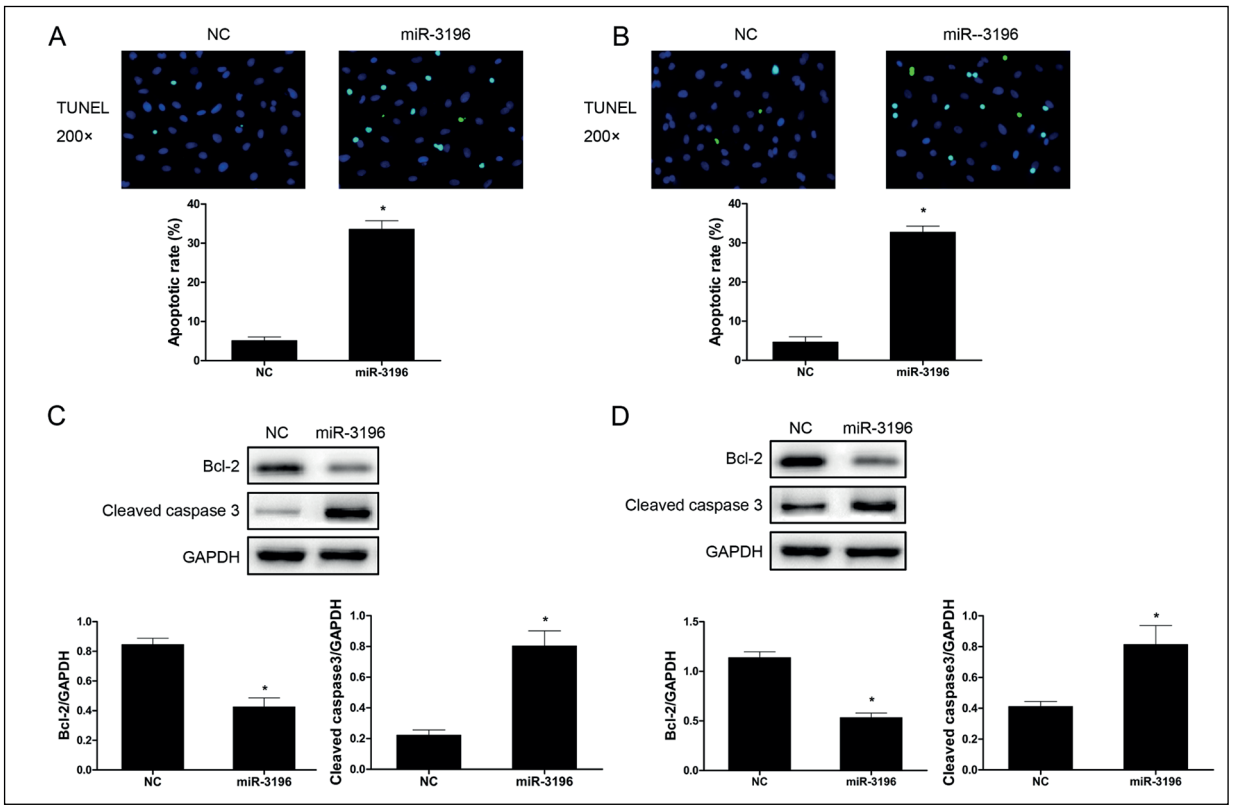

Figure 3. Overexpression of miR-3196 could induce breast cancer cell apoptosis. TUNEL assay was used to detect the apoptosis of ***A,*** (MCF-7) and ***B,*** (MDA-MB-231) cells (Magnification: 200×). The expression levels of Bcl-2 and Caspase-3 in ***C,*** (MCF-7) and ***D,*** (MDA-MB-231) cells were detected by western blot analysis. $*p < 0.05$.

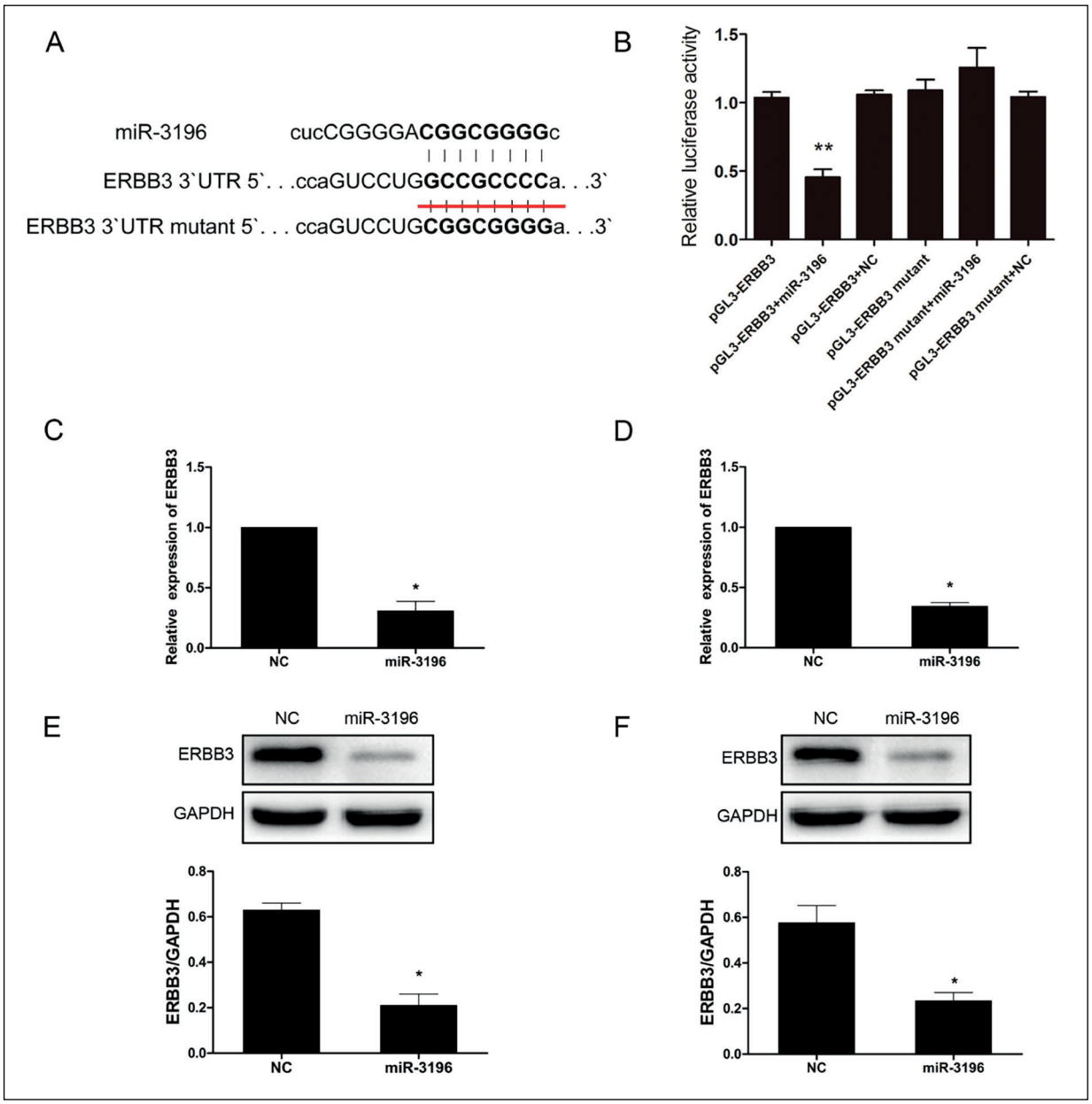

Figure 4. MiR-3196 targeted the 3'-UTR of ERBB3. ***A,*** The miRNA target prediction software starBase (http://starbase.sysu.edu.cn/) was used to screen the potential target genes of miR-3196. The potential binding site predicted by starBase database was exhibited. ***B,*** A luciferase reporter gene assay was conducted to validate this interaction. ***C-F,*** Both the mRNA and protein levels of ERBB3 were remarkably reduced by miR-3196 up-regulation. *$p < 0.05$.

Meanwhile, the global incidence of breast cancer has been on the rise since the late 1970s. Thus, breast cancer has become a major public health problem in the current society. Aberrant expression of miRNAs in different cancers has been widely reported. In breast cancer, low expression of miR-590 regulates cell invasion and epithelial-mesenchymal transition *via* influencing Wnt-β-catenin signaling pathway[9]. MiR-1254 functions as an oncogene through regulating RASSF9 in breast cancer[10]. Meanwhile, miR-19b acts as a prognostic biomarker, which may also enhance cancer progression *via* PI3K/AKT signaling pathway[11]. Wang et al[12] have found that miR-3196 is lowly expressed in breast cancer through miRNA microarray. Moreover, Pena-Chilet et al[13] reported that miR-3196 is significantly reduced in the microRNA profile of young breast cancer patients.

Here, we demonstrated that miR-3196 was remarkably decreased in breast cancer tissues when compared with adjacent normal tissues. Our findings were consistent with the above two studies. Furthermore, overexpression of miR-3196 could repress the proliferation and induce the apoptosis of breast cancer cells. After overexpressing miR-3196, the expression levels of Cyclin D1, Ki67 and Bcl-2 were significantly reduced, whereas Caspase-3 was significantly increased. ERBB3 is a member of the epidermal growth factor receptor (EGFR) family, encoding receptor tyrosine kinases. Upregulation of ERBB3 promotes the proliferation, migration and invasion of cervical cancer cells *via* interaction with MTK-1[14]. ErbB3 promotes lactogenic expansion and differentiation of mammary glands during pregnancy by activating Akt and STAT5A[15]. Previous investigations[16-18] have indicated that ERBB3 is significantly increased in breast cancer. Meanwhile, upregulation of ERBB3 is positively correlated with cancer metastasis, tumor size and recurrence. ERBB3 is also involved in the development of resistance to anti-estrogens and ErbB tyrosine kinase inhibitors, such as tamoxifen and gefitinib[16-18]. Recently, ERBB3 has been proposed to be related to acquired resistance to ErbB-targeted drugs[19]. In the present wowrk miRNA target prediction software starBase indicated that ERBB3 was a potential target for miR-3196. Subsequent RT-PCR, Western blot and luciferase reporter gene assay confirmed this suggestion. Meanwhile, both the mRNA and protein levels of ERBB3 were remarkably reduced after miR-3196 upregulation. All these findings suggested that miR-3196 targeted the 3'-UTR of ERBB3.

Conclusions

We found that the miR-3196 was lowly expressed in breast cancer tissues. Overexpression of miR-3196 could repress the proliferation and induce the apoptosis of breast cancer cells. Moreover, miR-3196 targeted the 3'-UTR of ERBB3. We validated the role of miR-3196 in breast cell proliferation and apoptosis. Furthermore, this study might provide novel insights into the underlying biomarker and therapeutic target for breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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