

ORIGINAL ARTICLE

Circular RNA-100219 promotes breast cancer progression by binding to microRNA-485-3p

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Summary

Purpose: To investigate whether circ-100219 could promote the proliferation and migration of breast cancer cells by up-regulating NTRK3 after binding to microRNA-485-3p, thus participating in the development of breast cancer.

Methods: Breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-549 and human mammary epithelial cells were cultured. The expression levels of circ-100219, microRNA-485-3p and NTRK3 in breast cancer and paracancer tissues were determined using real-time quantitative polymerase chain reaction (RT-qPCR). The regulatory effects of circ-100219, microRNA-485-3p and NTRK3 on the proliferative and migratory capacities of breast cancer cells were assessed using cell counting kit-8 (CCK-8) and Transwell assay, respectively. Dual-luciferase reporter gene assay was conducted to determine the binding condition among circ-100219, microRNA-485-3p and NTRK3. Rescue experiments were performed in co-transfected breast cancer cells.

Results: RT-qPCR data showed that circ-100219 and NTRK3 were highly expressed, whereas microRNA-485-3p was lowly expressed in breast cancer tissues than those of

paracancer tissues. Knockdown of circ-100219 in MCF-7 and MDA-MB-231 cells inhibited their proliferative and migratory capacities. On the contrary, microRNA-485-3p knockdown improved the proliferative and migratory capacities. Dual-luciferase reporter gene assay revealed that circ-100219 could bind to microRNA-485-3p and NTRK3 was the target gene of microRNA-485-3p. Western blot results elucidated that circ-100219 stabilized NTRK3 expression, whereas microRNA-485-3p degraded NTRK3 expression. Rescue experiments demonstrated that overexpression of NTRK3 could partially reverse the inhibited proliferative and migratory capacities induced by circ-100219 knockdown in MCF-7 and MDA-MB-231 cells.

Conclusions: Overexpression of circ-100219 promotes the proliferative and migratory capacities of breast cancer cells by sponging microRNA-485-3p to upregulate the NTRK3 expression.

Key words: breast cancer, circ-100219, microRNA-485-3p, migration, NTRK3, proliferation

Introduction

In recent years, although early diagnosis and targeted therapies have greatly reduced the mortality of breast cancer patients, the recurrence, metastasis and radiochemotherapy resistance are still inevitable problems in the clinical treatment of breast cancer [1]. Studies have found that breast cancer is a result of multiple gene dysregulations, confirming that the occurrence and development of

breast cancer are influenced by intracellular gene network system [2].

Based on the length and structure, noncoding RNAs (ncRNAs) can be divided into long noncoding RNA (lncRNA), microRNA (miRNA), and newly discovered circular RNA (circRNA) [3]. These ncRNAs exert crucial biological functions in terms of their sequential or structural characteristics [4,5].

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Structurally, miRNAs are about 21-23 nucleotides in length [6]. LncRNA contains more than 200 nucleotides, and some of them even have more than 100,000 nucleotides [7]. As a novel ncRNA, circRNA presents a cyclized structure without polarity and polyA tail. Functionally, miRNAs regulate protein expressions by directly inhibiting their target genes [8]. It acts as a regulator and a DNA polymerase II inhibitor during transcription, and can participate in the processing and translation of mRNA at post-transcriptional level [9,10].

In contrast, circRNA currently is less studied. Unlike common RNA, circRNA possesses a closed loop structure and has a very high abundance in the eukaryotic transcriptome [4]. Most of the circRNAs are transcribed from exons, and their sequences are highly conserved among different organisms. CircRNAs have the characteristics of tissue specificity and expression specificity at different stages [11], since nucleases could not degrade circRNAs, allowing them to be more stable in cells than traditional linear RNAs. As a competitive endogenous RNA (ceRNA) in the cytoplasm, circRNA abolishes the inhibited target genes by sponging these miRNAs [12]. Current researches have found some vital circRNAs in breast cancer, such as circ-0001982, circ-ABCB10, circ-0008039, and circ-0001785 [13-16]. A previous study showed high expression of circ-100219 in breast cancer tissues, which predicts poor tumor prognosis [17]. The specific mechanism of circ-100219 in regulating breast cancer development, however, is still unclear.

In this study, we explored the biological function of circ-100219 and first proposed the effect of circ-100219/microRNA-485-3p/NTRK3 regulatory network on breast cancer progression.

Methods

Sample collection

Breast cancer and paracancer tissues were surgically resected from 48 patients admitted to the Affiliated Zhongshan Hospital of Dalian University from 2014 to 2017. All cases were pathologically diagnosed as breast cancer. Paracancer tissues were resected 5 cm away from the tumors without infiltration of tumor cells. All samples were cut into 1 cm³, washed with DEPC-PBS and preserved in liquid nitrogen. This study was approved by the ethics committee of the Affiliated Zhongshan Hospital of Dalian University. Signed informed consents were obtained from all participants before the study entry.

Cell culture and transfection

MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-549 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) (HyClone, South

Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained at 37°C, 5% CO₂. Cells were seeded in 6-well plates and cell transfection was performed until 50-60% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). We authorized GenePharma (Shanghai, China) to construct plasmids used in the present study.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) using Primescript RT Reagent (TaKaRa, Tokyo, Japan). RT-qPCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Tokyo, Japan), and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study were as follows: Circ-100219 (F: 5'-TGCTACAGACGACTCAGAGA-3', R: 5'-AGATGATGAAGGTGGTGCA-3'), MicroRNA-485-3p (GTCATACACGGCTCTCTCTCT), NTRK3 (F: 5'-TGAGAACCCCCAGTACTTCC-3', R: 5'-CTGAAACCATGTGACCTTGG-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'); U6 (F: 5'-CTCGCTTCGGCAGCAGCATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3').

Cell counting kit-8 (CCK-8) assay

Cells were plated into 96-well plates at a number of 1×10⁶/mL. After cell culture for 24 hrs, 10 µL of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well. Two hrs later, the optical density (OD) value at the wavelength of 450 nm was measured using a microplate reader.

Transwell assay

1×10⁵ transfected cells were seeded in the upper chamber. Five hundred µL of RPMI-1640 medium containing 10% FBS were added to the lower chamber. After 48 hrs of cell culture, the chamber was removed. Cells were fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet for 15 min. The inner layer cells were carefully removed. Penetrating cells were captured in 5 randomly selected fields of each sample.

Dual-luciferase reporter gene assay

Circ-100219-WT 3'UTR, circ-100219-MUT 3'UTR, NTRK3-WT 3'UTR or NTRK3-MUT 3'UTR were constructed respectively. Cells were seeded in the 24-well plates with 5×10⁵ cells per well and were co-transfected with 0.12 µg vector and 40 nM microRNA-485-3p mimic or negative control for 48 hrs. Luciferase activity was finally determined.

Western blot

Total protein was extracted using a cell lysate (RIPA) (Beyotime, Shanghai, China). Protein samples were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). Membranes were then blocked with

blocking solution for 1 hr. Primary antibody was used for incubation at room temperature for 2 hrs. After washing with tris buffered saline-tween (TBST), the corresponding secondary antibody was used for incubation for 2 hrs at room temperature. Enhanced chemiluminescence was performed to visualize immunoreactive bands.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) and Graphpad (La Jolla, CA, USA) were used for data analyses. Data were expressed as mean \pm standard deviation. Continuous variables were analyzed using the t-test, and categorical variables were analyzed using χ^2 test. $P < 0.05$ was considered to be statistically significant.

Results

Circ-100219 was highly expressed in breast cancer tissues

The expression of circ-100219 in 48 pairs of breast cancer and paracancer tissues was detected by RT-qPCR. The results showed that circ-100219 was highly expressed in breast cancer tissues (Figure 1A). Besides, its expression was significantly higher in advanced-stage breast cancer tissues (stage III-IV) than those in early stages (stage I-II), suggesting that circ-100219 may be

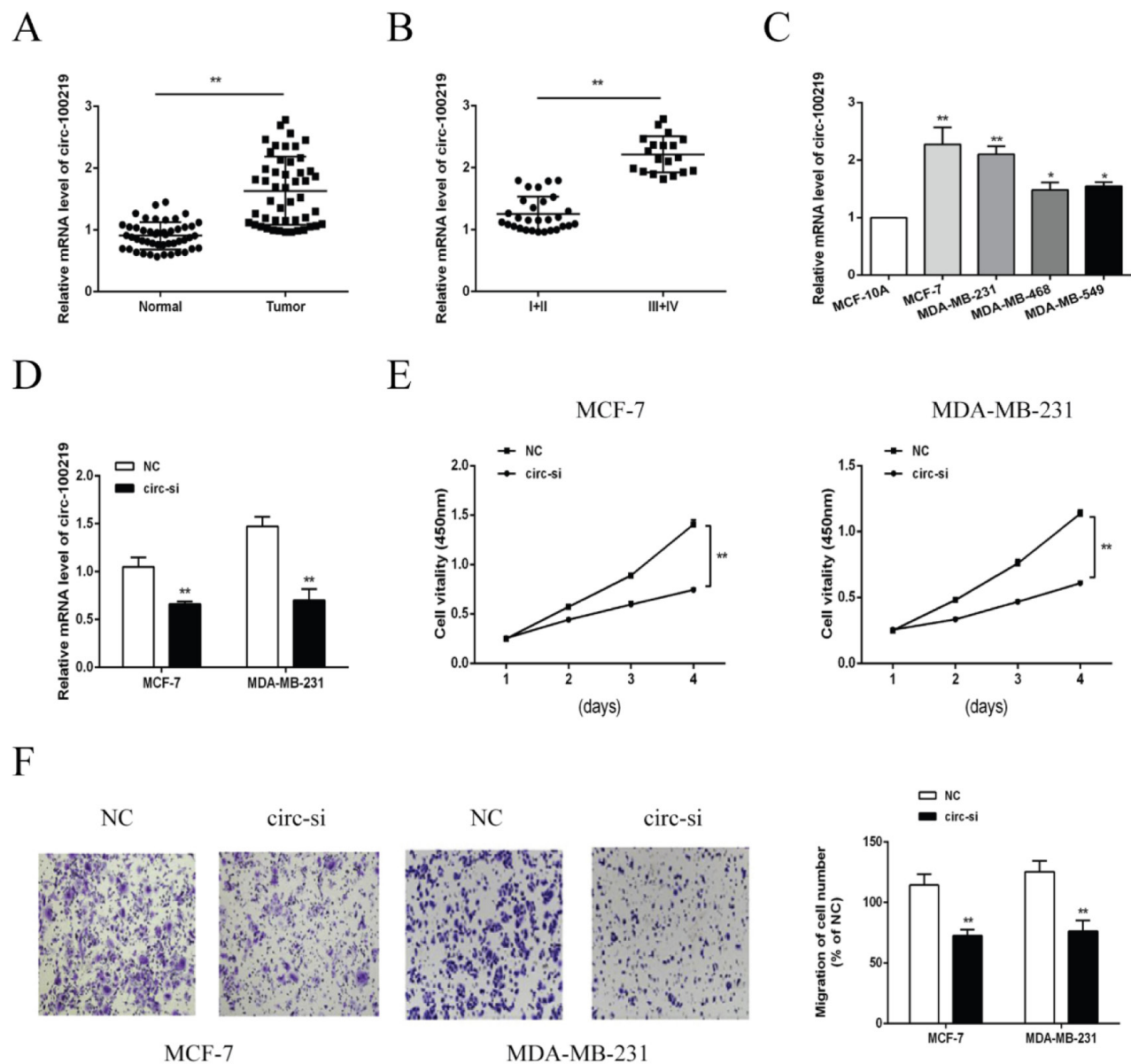


Figure 1. Circ-100219 knockdown inhibited proliferation and migration of breast cancer cells. **A:** Circ-100219 was highly expressed in breast cancer tissues. **B:** Circ-100219 was highly expressed in breast cancer tissues with stage III-IV than those with stage I-II. **C:** Circ-100219 was highly expressed in breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-549. **D:** Circ-100219 expression in MCF-7 and MDA-MB-231 cells after interference with circ-100219. **E:** Circ-100219 knockdown inhibited proliferation of breast cancer cells. **F:** Circ-100219 knockdown inhibited migration of breast cancer cells. * $p < 0.05$, ** $p < 0.01$.

involved in breast cancer development (Figure 1B).

Subsequently, we found that circ-100219 was also highly expressed in breast cancer cell lines (Figure 1C). Among them, the expression difference was pronounced in MCF-7 and MDA-MB-231 cells, which were selected for subsequent experiments.

Knockdown of circ-100219 inhibited proliferative and migratory capacities of breast cancer cells

After transfection of circ-100219 siRNA into MCF-7 and MDA-MB-231 cells, the expression of

circ-100219 was detected by RT-qPCR. It was found that circ-100219 siRNA transfection significantly inhibited the expression of circ-100219 in breast cancer cells (Figure 1D). CCK-8 results showed remarkable inhibition of proliferation after circ-100219 knockdown in MCF-7 and MDA-MB-231 cells (Figure 1E). We then examined the effect of circ-100219 on cell migration by Transwell assay. The results showed that knockdown of circ-100219 expression could attenuate the migration in breast cancer cells (Figure 1F).

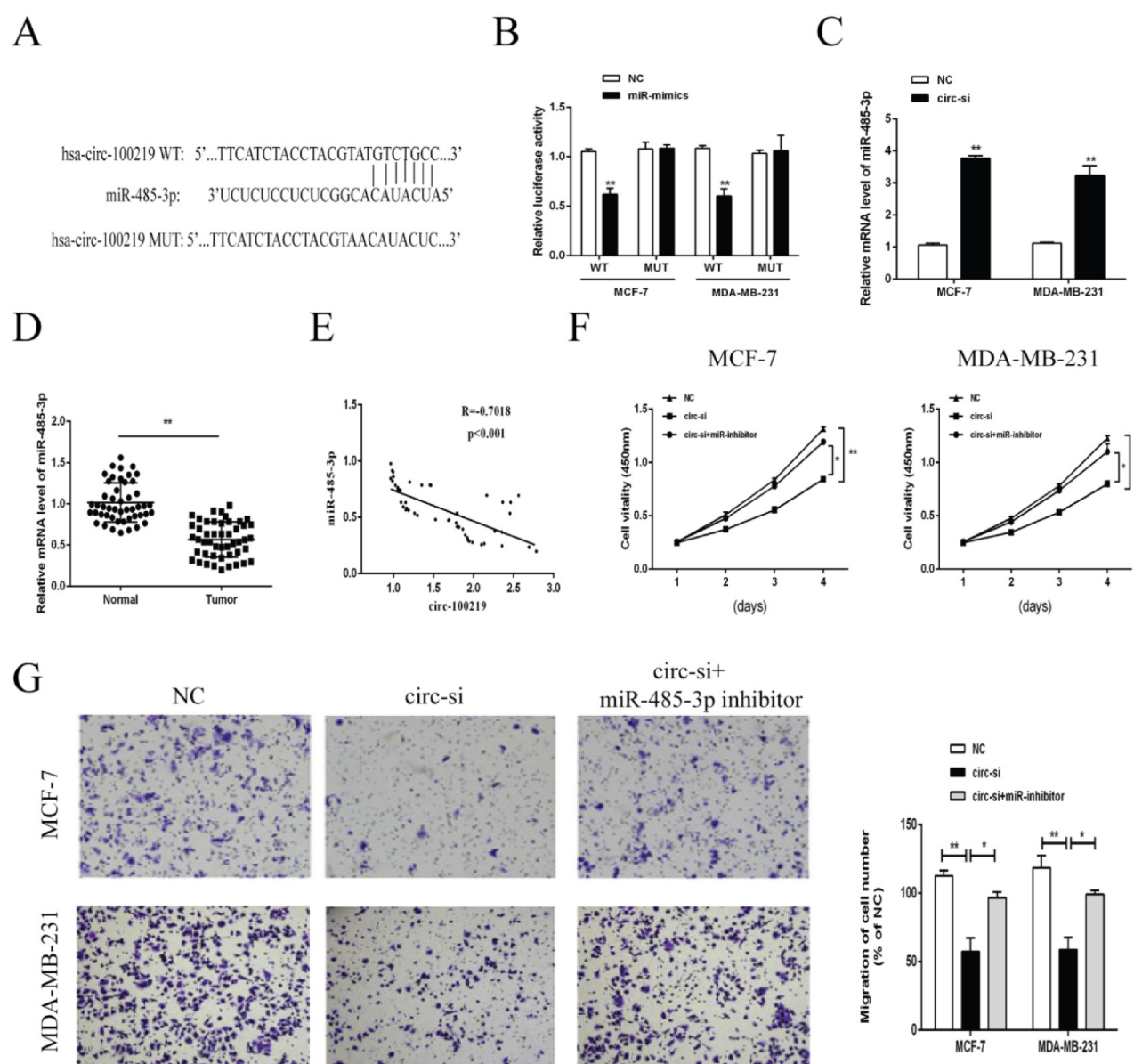


Figure 2. Circ-100219 regulated the proliferation and migration of breast cancer cells by binding to microRNA-485-3p. **A:** The binding site between circ-100219 and microRNA-485-3p predicted by bioinformatics. **B:** Dual-luciferase reporter gene assay verified that microRNA-485-3p could bind to circ-100219 3'-UTR. **C:** Circ-100219 knockdown upregulated microRNA-485-3p expression. **D:** MicroRNA-485-3p was lowly expressed in breast cancer tissues. **E:** A negative correlation between circ-100219 and microRNA-485-3p. **F, G:** The inhibited proliferation (**F**) and migration (**G**) of MCF-7 and MDA-MB-231 cells induced by circ-100219 knockdown were partially reversed by microRNA-485-3p interference. * $p < 0.05$, ** $p < 0.01$.

Circ-100219 regulated proliferation and migration of breast cancer cells by binding to microRNA-485-3p

We predicted miRNAs that bind to circ-100219 through bioinformatics. Finally, microRNA-485-3p was screened out (Figure 2A). Dual-luciferase reporter gene assay showed decreased luciferase activity in MCF-7 and MDA-MB-231 cells of circ-100219-WT 3'UTR group. However, no significant change in luciferase activity was observed in circ-100219-MUT 3'UTR group (Figure 2B), indicating that microRNA-485-3p binds to circ-100219. After interference with circ-100219 in MCF-7 and MDA-MB-231 cells, microRNA-485-3p expression increased (Figure 2C). Subsequently, we examined the expression of microRNA-485-3p in breast

cancer and paracancer tissues by RT-qPCR. The data showed that microRNA-485-3p was highly expressed in breast cancer tissues (Figure 2D). By analyzing the expressions of circ-100219 and microRNA-485-3p in breast cancer tissues, a negative correlation was found between the two genes (Figure 2E). Subsequently, we examined the effects of microRNA-485-3p on cell proliferation and migration by CCK-8 and Transwell assay, respectively. The inhibited proliferation and migration of MCF-7 and MDA-MB-231 cells induced by circ-100219 knockdown were partially reversed by microRNA-485-3p interference (Figure 2F and 2G). The results indicated that circ-100219 may regulate proliferation and migration of breast cancer cells by binding to microRNA-485-3p.

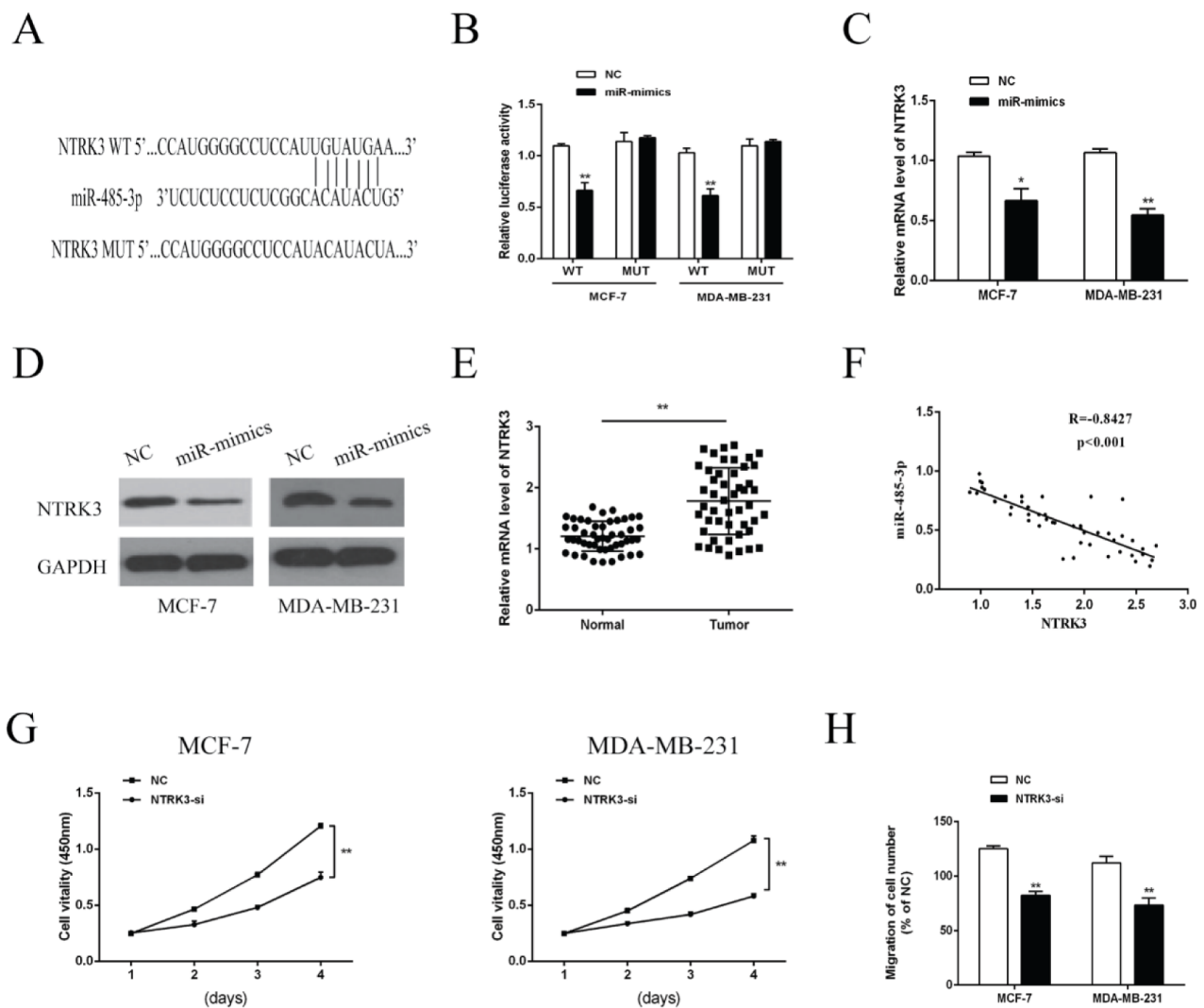


Figure 3. MicroRNA-485-3p targeted and degraded NTRK3. **A:** The binding site between microRNA-485-3p and NTRK3 predicted by bioinformatics. **B:** Dual-luciferase reporter gene assay verified that microRNA-485-3p could bind to UTRK3 3'-UTR. **C, D:** MicroRNA-485-3p overexpression downregulated mRNA (**C**) and protein (**D**) levels of NTRK3. **E:** NTRK3 was highly expressed in breast cancer tissues. **F:** Negative correlation between microRNA-485-3p and NTRK3. **G, H:** NTRK3 knockdown in MCF-7 and MDA-MB-231 cells inhibited cell proliferation (**G**) and migration (**H**). * $p < 0.05$, ** $p < 0.01$.

MicroRNA-485-3p targeted and degraded NTRK3

Through similar methods, NTRK3 was screened out as the target gene of microRNA-485-3p (Figure 3A). Dual-luciferase reporter gene assay showed decreased luciferase activity in MCF-7 and MDA-MB-231 cells of NTRK3-WT 3'UTR group. However, no significant change in luciferase activity was observed in NTRK3-MUT 3'UTR group (Figure 3B), indicating that microRNA-485-3p binds to NTRK3. Overexpression of microRNA-485-3p downregulated both mRNA and protein levels of NTRK3 in MCF-7 and MDA-MB-231 cells (Figure 3C and 3D). Subsequently, we examined the expression of NTRK3 in breast cancer and paracancer tissues by RT-qPCR. The data showed that NTRK3 was highly expressed in breast cancer tissues (Figure 3E). By analyzing the expressions of microRNA-485-3p and NTRK3 in breast cancer tissues, a negative correlation was found between the two genes (Figure 3F). Functional experiments showed inhibited proliferation and migration of MCF-7 and MDA-MB-231 cells induced by NTRK3 knockdown (Figure 3G and 3H).

Circ-100219 promoted the proliferative and migratory capacities of breast cancer cells by sponging microRNA-485-3p to upregulate NTRK3 expression

To further verify the regulatory relationship among circ-100219, microRNA-485-3p and NTRK3, we examined the expression of NTRK3 after MCF-7 and MDA-MB-231 cells were interfered with both circ-100219 and microRNA-485-3p. Interference with circ-100219 in MCF-7 and MDA-MB-231 cells downregulated NTRK3 expression. On the contrary, NTRK3 expression increased by co-transfection of si-circ-100219 and si-microRNA-485-3p (Figure 4A and 4B). By analyzing the expressions of NTRK3 and circ-100219 in breast cancer tissues, a significant positive correlation was found between the two genes (Figure 4C). Moreover, we tested the proliferative and migratory capacities after circ-100219 interference and NTRK3 overexpression in MCF-7 and MDA-MB-231 cells and found that circ-100219 knockdown in MCF-7 and MDA-MB-231 cells inhibited cell proliferation and migration, while overexpression of NTRK3 reversed the inhibited proliferation and migration (Figure 4D and 4E). These results indicated that circ-100219 may

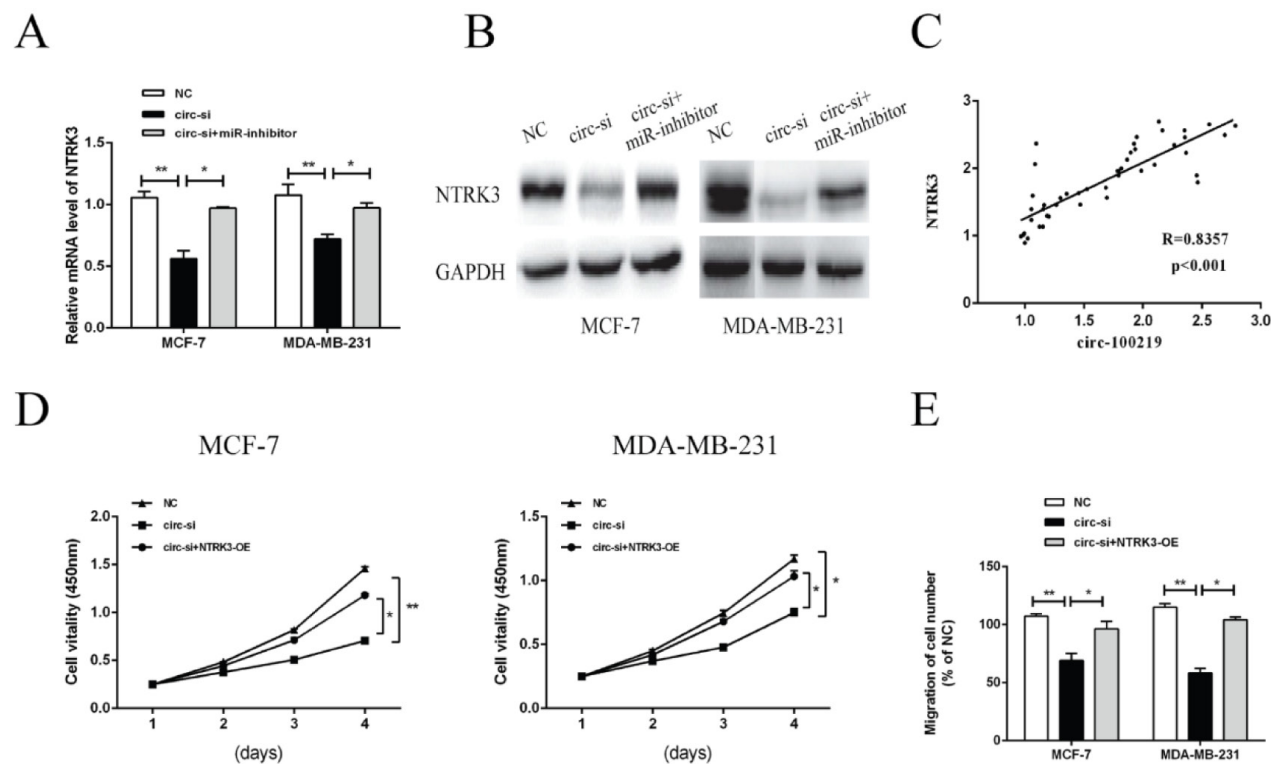


Figure 4. Circ-100219 promoted proliferative and migratory capacities of breast cancer cells by sponging microRNA-485-3p to upregulate NTRK3 expression. **A, B:** Interference with circ-100219 in MCF-7 and MDA-MB-231 cells downregulated NTRK3 expression. NTRK3 expression increased by co-transfection of si-circ-100219 and si-microRNA-485-3p. **C:** Significant positive correlation between NTRK3 and circ-100219. **D, E:** Circ-100219 knockdown in MCF-7 and MDA-MB-231 cells inhibited cell proliferation and migration, while overexpression of NTRK3 reversed the inhibitory effects. * $p<0.05$, ** $p<0.01$.

promote the proliferation and migration of breast cancer cells by upregulating NTRK3 expression after binding to microRNA-485-3p.

Discussion

CircRNA is linked by the 3' and 5' ends of the RNA molecule. Its sequential structure is not present in the genomic DNA, which is formed during the splicing of its transcription products. Hence, circRNA is detected by the primers designed, based on both ends of the junction. Due to lack of 3' and 5'-ends, circRNAs are more stable than most of linear RNAs since they are resistant to exonuclease-mediated degradation [18]. Many circRNAs are derived from protein-encoding genes, i.e., exon sequences of genes. However, most of circRNAs could not encode proteins, which are considered as a kind of ncRNAs. CircRNA is abundantly present in eukaryotic cells with certain organization, timing and disease specificities, and exerts an important regulatory role in the disease progression [19]. In this study we found that circ-100219 is highly expressed in breast cancer tissues and cell lines. Subsequent experiments suggested that circ-100219 knockdown inhibits the proliferative and migratory capacities of breast cancer cells. To investigate the mechanism by which circ-100219 affects the proliferation and migration of breast cancer cells, we searched for its target gene and NTRK3 was screened out.

NTRK3, also known as TrKc, is a neurotrophic receptor [20]. The neurotrophic receptors are essential growth factors involving the nervous system. There are four subtypes of neurotrophic receptors, namely nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic factor 3 (NT3) and NT4/5 [21]. Each factor has high affinity for a specific neurotrophic tyrosine kinase receptor [22]. NTRK3 is greatly involved in the occurrence, development and metastasis of tumors [23-27]. In our study, NTRK3 was highly expressed in breast

cancer cells. Knockdown of NTRK3 remarkably decreased the proliferative and migratory capacities of breast cancer cells.

To investigate the mechanism by which circ-100219 regulates NTRK3, we started searching for the "bridge" that connects circ-100219 and NTRK3. Studies have shown that NTRK3 can control cell proliferation by interacting with miRNAs. Meanwhile, circRNAs exert biological functions by regulating miRNA expressions [18]. We therefore hypothesized that circ-100219 may regulate NTRK3 by sponging the expressions of certain miRNAs. By base sequence analysis, microRNA-485-3p was found to be able to complementarily pair with both bases of circ-100219 and NTRK3 3'UTR. We speculated that circ-100219 may indirectly affect the expression of NTRK3 by regulating microRNA-485-3p. Our study showed that highly expressed circ-100219 can bind to microRNA-485-3p and inhibit its expression, thereby abolishing microRNA-485-3p-induced NTRK3 degradation. Furthermore, circ-100219 and NTRK3 could promote the proliferation and migration of breast cancer cells, while microRNA-485-3p inhibited cell proliferation and migration. It is concluded that circ-100219 may indirectly promote the expression of NTRK3 by inhibiting the expression of microRNA-485-3p, thereby affecting the proliferative and migratory capacities of breast cancer cells.

Conclusions

Overexpression of circ-10021 promoted the proliferative and migratory capacities of breast cancer cells by upregulating NTRK3 after sponging microRNA-485-3p. We propose that circ-100219 may possess diagnostic and therapeutic values on breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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