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CircRNA_100876 promote proliferation and metastasis of breast cancer cells through adsorbing microRNA-361-3p in a sponge form

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Abstract. – OBJECTIVE: This study was designed to investigate the expression level of circRNA_100876 in breast cancer (BC) tissues or cells, and to further explore whether it can promote cell metastasis and proliferative capacity via targeting microRNA- 361-3 p.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression of circRNA_100876 in 50 pairs of BC tissue specimens and corresponding adjacent ones, and the correlation between circRNA_100876 expression and prognosis of patients with BC was analyzed. Meanwhile, qRT-PCR was further performed to verify circRNA_100876 level in BC cell lines. In addition, circRNA_100876 knockdown model was constructed using lentivirus and transfected in BC cells. Subsequently, the impact of circRNA_100876 on BC cell function was analyzed using Cell Counting Kit-8 (CCK-8), transwell and clone formation assays. The interplay between circRNA_100876 and microRNA-361-3 p was verified using the Luciferase reporter gene assay and cell reverse experiment.

RESULTS: QRT-PCR results showed that circRNA_100876 level in BC tissues was conspicuously higher than that in the adjacent tissues, and the patients with distant metastasis had higher expression than those without. Moreover, patients with a high expression of circRNA_100876 had a relatively lower overall survival rate. Compared with the NC group, the cell proliferation and invasion ability of circRNA_100876 knockdown group was conspicuously decreased. QRT-PCR revealed that microRNA-361-3p and circRNA_100876 showed a negative correlation in the expression level of genes in BC tissues. In addition, the results of the Luciferase reporter gene assay confirmed that circRNA_100876 can be targeted by microRNA-361-3p through their binding site.

CONCLUSIONS: High expression of circRNA_100876 is conspicuously positively relevant to poor prognosis of BC patients. Additionally, circRNA_100876 is able to promote BC metastasis as well as proliferative capacity by modulating microRNA-361-3p expression.

Key Words:
CircRNA_100876, MicroRNA- 361-3p, BC, Proliferation, Metastasis.

Introduction

BC is a serious malignant tumor that has the highest morbidity among women and thus seriously threatens women's health and lives. More than 1.3 million women worldwide are diagnosed with BC every year, and more than 500,000 women die of BC[1-3]. With the rapid changes in lifestyle and deterioration of the ecological environment, the incidence of BC in China has shown a relatively significant upward trend--up to 3-4% of new cases each year, which make it become one of the most rapidly rising malignant tumors in China. Moreover, it tends to occur in younger populations, making the median age of Chinese BC female patients about a decade earlier than those in the west[4,5]. Therefore, expounding the pathogenesis of BC, controlling the incidence of BC, improving the therapeutic effect of BC and reducing BC mortality have become major issues to be solved[6-8].

Circular RNAs (circRNAs) are closed circular RNAs formed by covalent bonds and abundant in the transcriptome of eukaryotic cells, which are different from traditional linear RNAs. They usually include exon sequences and are spliced at classical splicing sites[9,10]. The characteristics of this gene expression were

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first identified in humans and mice and soon found to be common in all eukaryotes studied by molecular biologists[11,12]. CircRNA was first found to be enriched in the brain and increased conspicuously during fetal development[12]. It is mainly produced by exons or exon sequences, and reverse complement or RNA-binding proteins are necessary for circRNA production[13,14]. Most circRNAs are conserved in species, stable and resistant to RNases, and often exhibit tissue-specific or developmental stage-specific expression[13-15]. Recent studies[15,16] have suggested that circRNA can function as a miRNA sponge, regulating splicing and transcription and modifying parental gene expression. CircRNA does not have a 5' end cap and a 3' end poly(A) tail, which is formed by reverse splicing by non-canonical splicing, and is abundantly present in the cytoplasm of eukaryotic cells, but there are also a small number of intron-derived circRNAs are present within nucleic acids. CircRNAs possess considerable organization, timing and disease specificity, and has a certain relationship with the occurrence and development of many diseases, including the metastasis and invasion of malignant tumors[11,16]. At present, there are few reports about circRNA_100876 in the development of tumor[17,18]. Bioinformatics database showed that circRNAs were highly expressed in breast cancer tissues, and circRNA_100876 specifically expressed in breast cancer was further selected as the research object.

Therefore, this study comprehensively analyzed the expression and biological role of circRNA_100876 in BC and its correlation with clinicopathological characteristics, and studied the interplay between circRNA_100876 and miR-361-3p expression, to further explore the molecular mechanism of BC regulation.

Patients and Methods

Patients and BC Samples

In this work, 50 pairs of breast tissue samples and adjacent normal ones were collected from surgically treated BC cases and then stored at -80°C. This study was approved by the Ethics Committee of Nanchang University. Patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Cell Lines and Reagents

The human BC cell lines (MCF-7, MDA-MB-231 and SKBR3) and normal mammary epithelial cell line, MCF-10A, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured in DMEM medium containing 10% FBS at 37°C incubator with 5% CO_2.

Transfection

The negative control group (NC) and the lentivirus containing circRNA_100876 knockdown sequence (circRNA_100876-S1 or circRNA_100876-S2) were purchased from Shanghai GenePharma Company (Shanghai, China). The cells were plated in 6-well plates and grown to a cell density of 50%. After transfection was performed, cells were harvested 48 hours later for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assay

The cells after 48 h of transfection were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h and 96 h respectively, and then added with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, cells were collected. 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was replaced after one week, and then changed twice a week. After 2 weeks, the cells were cloned, the medium was discarded, and the cells were washed twice with PBS. Afterward, cells were fixed in 2 mL of methanol for 20 minutes. After the methanol was removed, cells were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), and then stained with 0.1% crystal violet staining solution for 20 minutes. Lastly, the cell sample was photographed and counted under a light-selective environment.

Transwell Cell Migration Assay

After transfection for 48 hours, the cells were digested, centrifuged and resuspended in a medium without FBS to adjust the density to 5×10^5 cells/mL. A cell suspension of 200 uL (1×10^5 cells) was added to the upper chamber, and 700 uL of a medium containing 20% FBS was added to the lower chamber. According to the different migration abilities of each cell line, the cells were then put back into the incubator and continued to culture for a specific time. The transwell chamber was clipped, washed 3 times with 1 x PBS, and placed in methanol for 15 min cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, Real Time-Polymerase Chain Reaction (RT-PCR) was performed according to SYBR® Premix Ex TaqTM (Takara, Otsu, Shiga, Japan) kit instructions, and the PCR reaction was performed using the StepOne Plus Real Time-PCR System. Samples were run in triplicate and the expression was normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes for human. The Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data with the software iQ5 2.0. The gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers used are listed as followed: circRNA_100876 forward: 5'-CTGGTGCAGTGGAAGCAGAG-3'; Reverse: 5'-CGACCCTCCATTGCTCTTCT-3'. GAPDH forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3'; Reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'. miRNA-361-3p forward: 5'-TGCGGTAAGGCACGCGGGAAT-3'; Reverse: 5'-GGTACAAACAGGGAGGGA-3'. U6 forward: 5'-ATGGCTATAAATAGATACACGC-3'; Reverse: 5'-GGTACAAACAGGGAGGGA-3'.

Dual-Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the Luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter gene plasmid into MCF-7 and SKBR3 cells or other related cell lines. If the transcription factor activates the target promoter, the Luciferase gene is expressed, and the amount of Luciferase expression is directly proportional to the intensity of the transcription factor. A specific Luciferase substrate was added, and Luciferase reacted with the substrate to generate fluorescence. By measuring the intensity of the fluorescence, the activity of the Luciferase was determined to figure out whether the transcription factor can interact with the target promoter fragment.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and error bars are mean ± standard deviation ($\bar{x}$±SD). $p<0.05$ was considered statistically significant.

Results

CircRNA_100876 Was Conspicuously Increased in BC Tissues and Cell Lines

To determine the expression characteristics of circRNA_100876 in BC, qRT-PCR was used to detect the difference in the expression of circRNA_100876 in BC tissues and adjacent tissues. The results showed that circRNA_100876 was elevated in BC tumor tissues compared with the adjacent tissues (Figure 1A), suggesting that circRNA_100876 may act as an oncogene in BC. In addition, we compared the expression of circRNA_100876 in patients with or without distant metastases and found that patients with distant metastases had conspicuously higher levels of circRNA_100876 expression than those without (Figure 1B). Compared to the normal mammary epithelial cell line (MCF-10A), circRNA_100876 was conspicuously highly expressed in BC cells (Figure 1C). Moreover, to explore the relation-

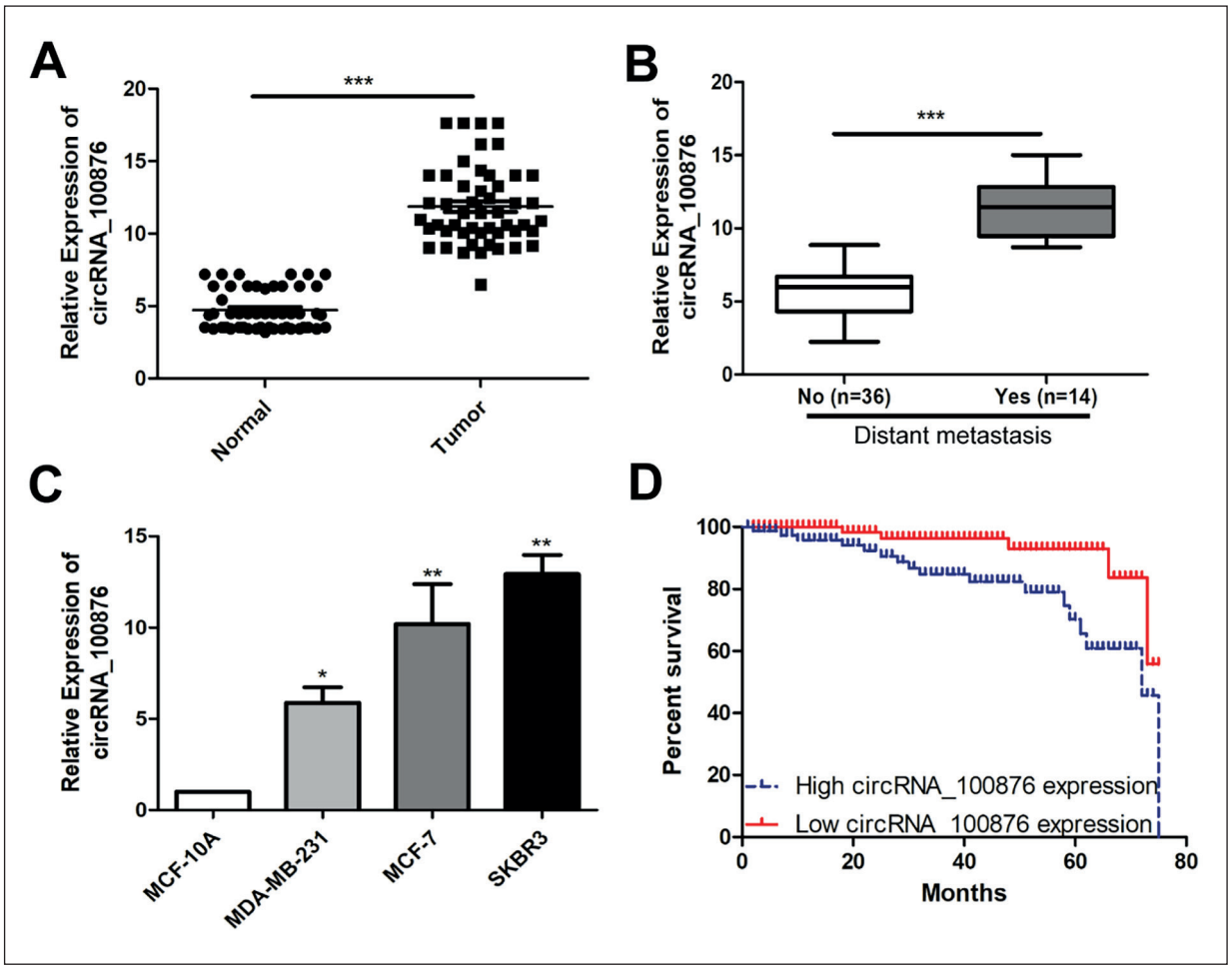

Figure 1. CircRNA_100876 was highly expressed in breast cancer tissues and cell lines. ***A,*** qRT-PCR detection of differential expression of circRNA_100876 in breast cancer tissues and adjacent tissues. ***B,*** qRT-PCR detection of differential expression of circRNA_100876 in BC tumor tissues with distant metastasis. ***C,*** qRT-PCR detection of expression level of circRNA_100876 in breast cancer cell lines. ***D,*** The Kaplan-Meier survival curve of breast cancer patients based on circRNA_100876 expression showed that patients with high expression group had significantly worse prognosis than low expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

ship between the expression of circRNA_100876 and the prognosis of BC patients, we collected relevant follow-up data. The Kaplan-Meier survival curves showed that high expression of circRNA_100876 was conspicuously associated with poor prognosis of BC. The higher the expression level of circRNA_100876, the worse the prognosis (p<0.05; Figure 1D).

Silencing CircRNA_100876 Inhibited Metastasis and Proliferative Capacity of BC Cells

To investigate the cytological function of circRNA_100876 in BC, MCF-7 and SKBR3 cell lines with circRNA_100876 knocked down were constructed and qRT-PCR was performed to verify the interference efficiency. The results were statistically significant (Figure 2A). In the MCF-7 and SKBR3 cell lines, circRNA_100876 was knocked down, and CCK-8, transwell and colony formation assays were used to detect cell proliferation, invasion and migration. The results of CCK-8 and colony formation experiments showed that the cell proliferation ability of circRNA_100876-silencing group was significantly lower than that of the NC group (Figure 2B, 2C). In addition, transwell results revealed that the ability of BC cells to invade and migrate after silencing circRNA_100876 decreased significantly (Figure 2D).

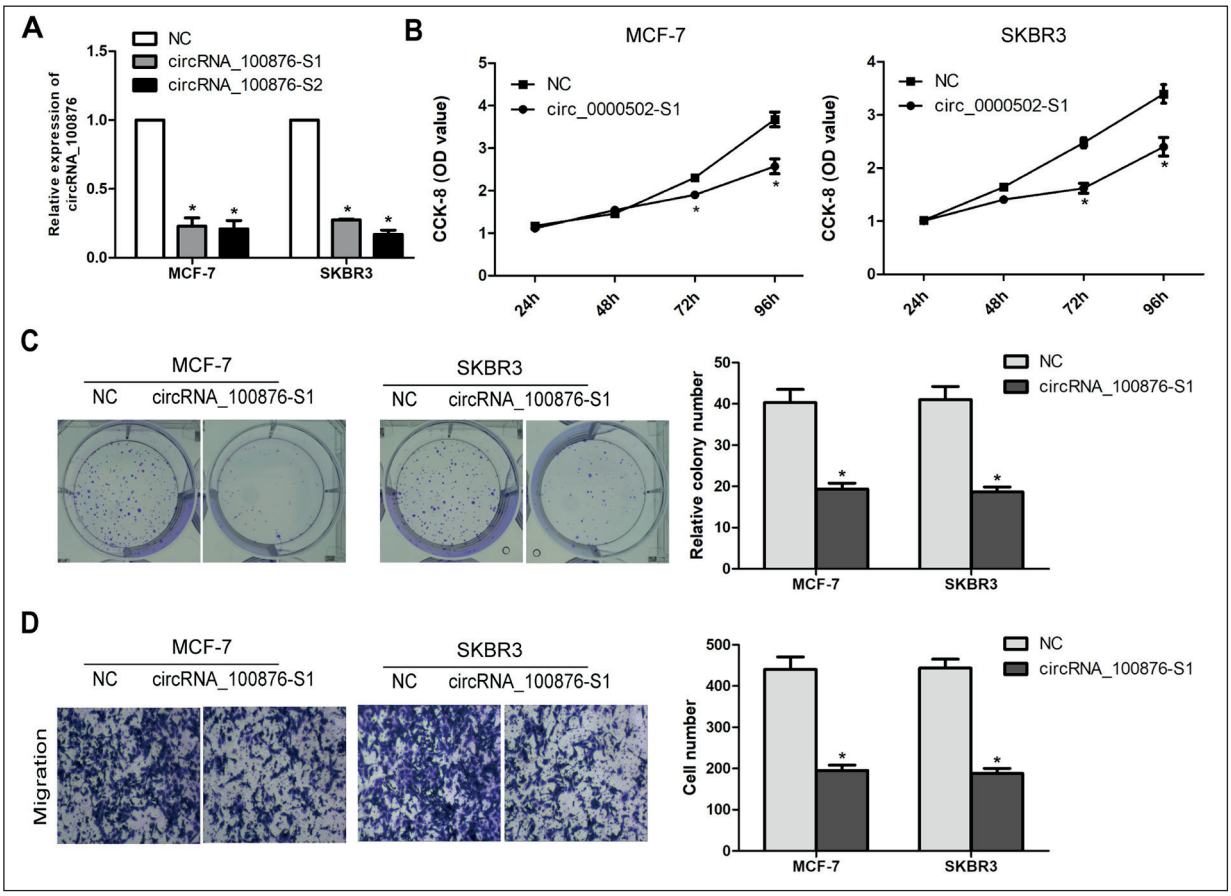

Figure 2. Silencing circRNA_100876 inhibited breast cancer cell proliferation and metastasis. ***A,*** qRT-PCR verified the interference efficiency of si-circRNA_100876 in MCF-7 and SKBR3 cell lines. ***B,*** CCK-8 assay was used to detect the effect of silencing circRNA_100876 in McF-7 and SKBR3 cell lines on BC cell proliferation. ***C,*** Clonal formation assay was used to detect the effect of silencing circRNA_100876 on proliferation of breast cancer cells in MCF-7 and SKBR3 cell lines. ***D,*** The transwell assay was used to detect the effect of silencing circRNA_100876 on migration of breast cancer cells in MCF-7 and SKBR3 cell lines (Magnification: 20X). Data are mean ± SD, *$p<0.05$.

CircRNA_100876 can Adsorb MiRNA-361-3p in Sponge Form

To further verify the targeting effect of mir-361-3p on circRNA_100876, the Luciferase reporter gene assay was performed and the result demonstrated that circRNA_100876 can be indeed targeted by microRNA-361-3p *via* this binding site (Figure 3A). QRT-PCR experiments detected the microRNA-361-3p expression in 50 pairs of BC tissues and their corresponding adjacent tissues, as well as in BC cell lines. The results showed that the expression level of miRNA-361-3p in BC tissue was markedly lower than that in the adjacent tissues, and the difference was statistically significant (Figure 3B). The similar result was observed in breast cell lines (Figure 3C). Subsequently, circRNA_100876 and miR-361-3p were found to be negatively correlated in BC tissues (Figure 3D). In addition, to explore the relationship between the expression of microRNA-361-3p and the prognosis of BC patients, we collected relevant follow-up data. The Kaplan-Meier survival curves showed that low expression of microRNA-361-3p was significantly associated with poor prognosis of BC. The lower the expression level of microRNA- 361-3 p, the worse the prognosis ($p<0.05$; Figure 3E).

Overexpression of MicroRNA-361-3p Inhibited Metastasis and Proliferative Capacity of BC Cells

To investigate the cytological function of microRNA- 361-3 p in BC, we constructed MCF-7 and SKBR3 cell lines that overexpressed microRNA-361-3p, and verified the interference efficiency by qRT-PCR (Figure 4A). After over-

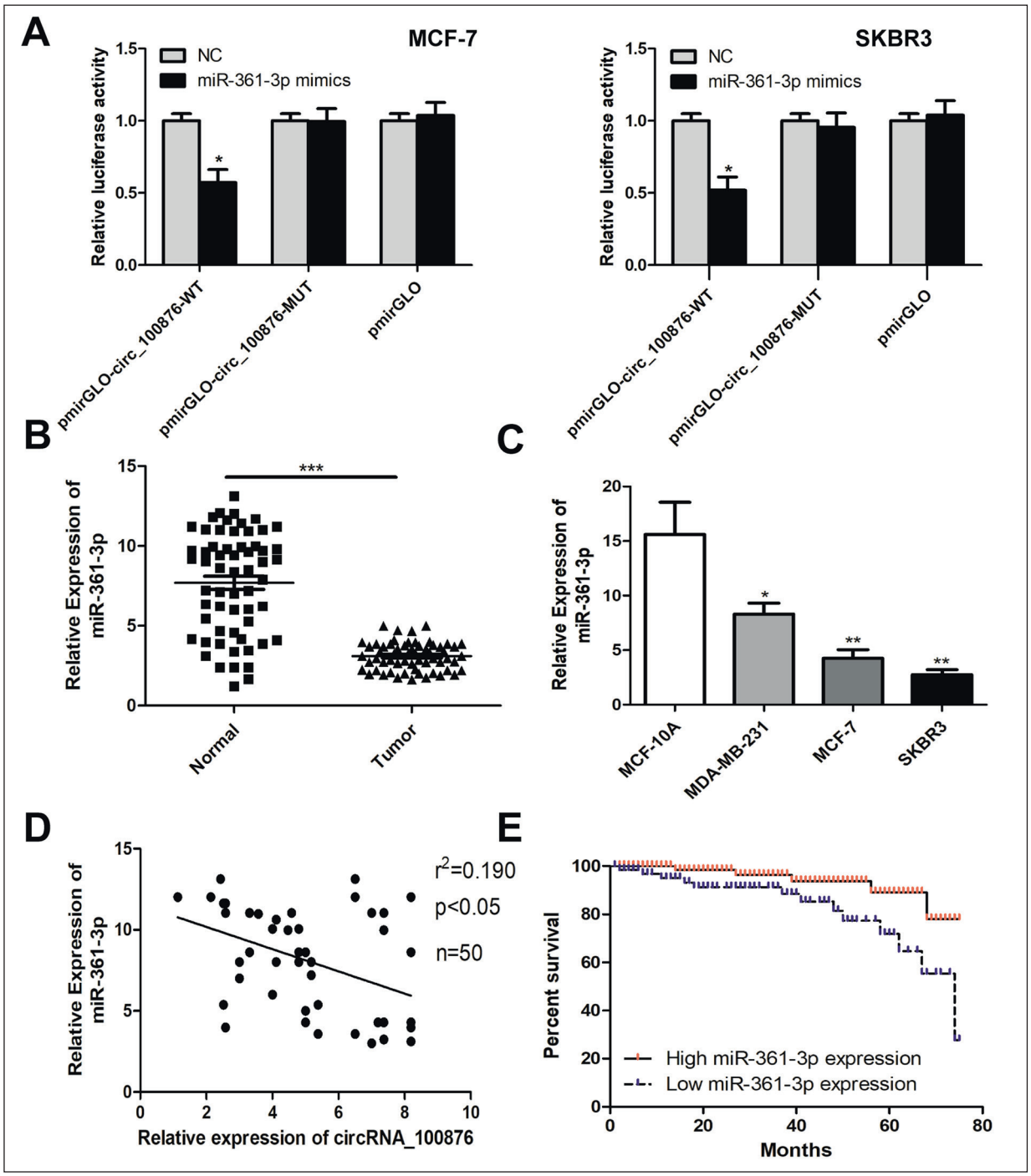

Figure 3. Direct targeting of miR-361-3p by circRNA_100876. ***A,*** The Dual-Luciferase reporter gene assay was used to verify the direct targeting of circRNA_100876 to miR-361-3p. ***B,*** qRT-PCR was used to detect the differential expression of miR-361-3p in breast cancer tissues and adjacent tissues. ***C,*** qRT-PCR was used to verify the mRNA expression level of miR-361-3p in breast cancer cell lines. ***D,*** There was a significant negative correlation between the expression levels of circRNA_100876 and miR-361-3p in breast cancer tissues. ***E,*** The Kaplan-Meier survival curve of breast cancer patients based on miR-361-3p expression indicated that the prognosis of patients with low expression was significantly worse than that of the high expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

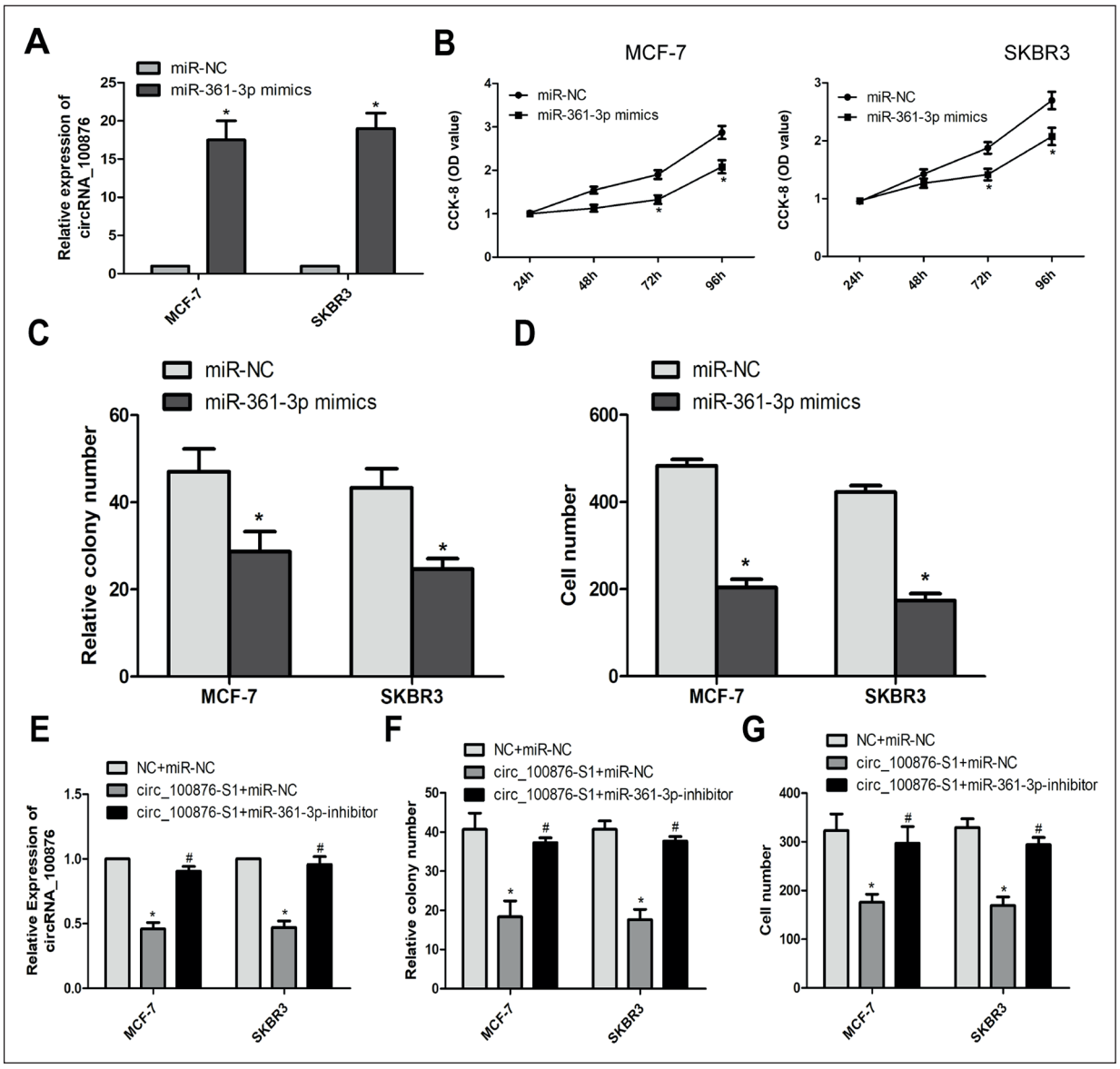

Figure 4. CircRNA_100876 regulated the expression of miR-361-3p in breast cancer tissues and cell lines. ***A,*** qRT-PCR was used to verify the efficiency of transfected miR-361-3p overexpression vector in MCF-7 and SKBR3 cell lines. ***B,*** CCK-8 assay was used to detect the influence of overexpression of miR-361-3p on breast cancer cell proliferation in MCF-7 and SKBR3 cell lines. ***C,*** Cloning assay was used to detect the influence of overexpression of miR-361-3p on breast cancer cell proliferation in MCF-7 and SKBR3 cell lines. ***D,*** The transwell assay was used to detect the influence of overexpression of miR-361-3p on invasion and migration of breast cancer cells in MCF-7 and SKBR3 cell lines. ***E,*** The expression levels of circRNA_100876 in cell lines co-transfected with circRNA_100876 and miR-361-3p were determined by qRT-PCR. ***F,*** The transwell assay was used to detect the role of clone formation of circRNA_100876 and miR-361-3p in regulating invasion and migration of breast cancer cells. ***G,*** The transwell assay was used to detect the role of co-transfection of circRNA_100876 and miR-361-3p in regulating invasion and migration of breast cancer cells. Data are mean ± SD, $*^{\#}p < 0.05$.

expressing microRNA- 361-3p in MCF-7 and SKBR3 cell lines, CCK-8, transwell and clone formation assays were used to detect cell proliferation, invasion and migration. The results of CCK-8 and colony formation experiments indicated that compared with miR-NC, the cell proliferation ability of cells in miR-361-3p mimics group was significantly reduced (Figure 4B, 4C). In addition, the invasion and migration ability of BC cells in microRNA-361-3p mimics group was found significantly inhibited (Figure 4D).

CircRNA_100876 Modulated MicroRNA-361-3p Expression and Promoted Malignant Progression of BC

To further explore the interaction between circRNA_100876 and microRNA- 361-3 p in BC cells, we silenced microRNA-361-3p in a cell line with low expression of circRNA_100876 in BC cells to investigate their role in BC. The qRT-PCR assay detected the transfection efficiency of circRNA_100876 after co-transfection (Figure 4E). Subsequently, we performed transwell and clone formation assay, and found that microRNA-361-3p can counteract the effect of circRNA_100876 on proliferation and invasion and migration of BC cells (Figure 4F and 4G).

Discussion

With the improvement of living standards and the westernization of living habits, the incidence of BC in China has increased year by year, and it has become one of the common malignant tumors with high incidence[3,5]. Surgery combined with radiotherapy and chemotherapy is the most commonly used treatment, which can undoubtedly be able to conspicuously prolong the disease-free survival time of BC patients, but this advantage does not change BC as the most common cause of death in women[8,11]. Among the direct causes of death in BC patients, recurrence and metastasis of tumor cells are the most common[1,3]. Therefore, it is an important issue to find more effective treatments and therapeutic drugs for breast cancer.

As closed circular RNA with stable expression, CircRNA is abundantly present in the eukaryotic transcriptome[11-13,15]. Studies[11-13,15] have shown that circRNA can act as a miRNA sponge in different species, that is, competing endogenous RNA (ceRNA) can competitively bind to miRNA and regulate the expression of target genes. The 3' and 5' ends conventionally present in linear RNA molecules are joined in a circular RNA to form a closed circular structure[19]. Classical RNA detection methods generally only identify RNA molecules with a PolyA "tail" structure, so circular RNA has often been ignored in the previous studies[19]. Most circRNAs are composed of exon sequences, which are conserved in different species, and their expression is tissue-specific and time-specific[9,13]. Because circular RNA is not sensitive to nucleases, it is more stable than linear RNA, which makes circular RNAs have considerable advantages in the development and application of new clinical diagnostic markers[9,13]. Although a considerable part of circRNA is derived from protein-coding genes, it has not been proven that circRNA can encode proteins in cells[12,15,20]. Cyclic RNA is therefore defined as a new class of non-coding RNAs that play a pivotal role in the fine-tuning of miRNA expression levels by competitively binding miRNAs for gene expression regulation[20]. In this study, CircRNA_100876 was selected as a candidate for relevant circRNA related to the malignant progression of BC, and the relationship between circRNA_100876 and the development of BC was finally determined in the knockdown cell line established using lentivirus. We have found through tissue validation that the expression of circRNA_100876 in BC tissues is conspicuously higher than that in adjacent tissues and is associated with poor prognosis of BC. Therefore, we believe that circRNA_100876 may play a role in promoting cancer in BC. To further study the molecular mechanism of circRNA_100876 in the development of BC to prove that circRNA_100876 is a disease-related gene, *in vitro* cell experiments were carried out. Subsequently, CCK-8 assay, transwell assay and colony formation assay were performed. It was found that MCF-7 and SKBR3 cell lines silencing circRNA_100876 can inhibit the proliferation, invasion and migration of BC cells compared with the NC group.

Cyclic RNA may play a critical role in disease regulation by competitive binding disease-associated miRNAs, and this circRNA-miRNA axis is a known regulatory model for several cancer-related pathways[10,16,19,20]. Cyclic RNA is a new member of the ceRNA family. Unlike linear RNA, circular RNA has no 5' end cap structure and 3' terminal polyadenylation (polyA) tail structure. Instead, it forms a closed ring structure connected end to end by covalent bonds, natural and widely found in eukaryotic cell lines of different races[9,15,17]. Therefore, an in-depth study of important signaling pathways and expression profiles of node molecules in the development of BC will greatly promote the development of BC diagnosis and treatment, and has high scientific research value. The results of this experiment show that miRNA-361-3p is less expressed in BC tumor tissues than in adjacent tissues, and miRNA-361-3p can inhibit proliferation, invasion and migration in BC cells. Bioinformatics was used to analyze the circRNA_100876 sequence containing a miRNA-361-3p binding site, and

the direct binding of circRNA_100876 to downstream miRNA-361-3p was verified by molecular biology experiments such as Dual-Luciferase reporter gene. The circRNA_100876 vector that deleted the miRNA-361-3p binding site failed to enrich miRNA-361-3p, further confirming the binding site of circRNA_100876 to miRNA-361-3p. The expression levels of circRNA 100876 and microRNA- 361-3p were found just negatively correlated in BC cell lines. In addition, the results of our recovery experiments in breast cancer cells suggested that circRNA_100876 could inhibit the expression of miRNA-361-3p, thereby promoting the metastasis and proliferative capacity of BC cells.

Conclusions

We detected that highly expressed circRNA_100876 was positively associated with poor prognosis of BC patients. In addition, circRNA_100876 can accelerate proliferation and invasion and migration of BC cells by regulating microRNA-361-3p expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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