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MicroRNA-548c-5p inhibits the proliferation of breast cancer cells through regulating Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the function of microRNA-548c-5p in breast cancer (BCa) and the underlying mechanism. Our findings might help to provide a theoretical basis for the diagnosis and treatment of BCa.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression level of microRNA-548c-5p in BCa tumor tissues and para-cancerous tissues. The relationship between microRNA-548c-5p expression and clinical indicators of BCa was analyzed. Meanwhile, the expression of microRNA-548c-5p in the BCa cells was detected by qRT-PCR as well. MicroRNA-548c-5p overexpression and the knockdown models were constructed in BCa cell lines MCF-7 and MDA-MB-231. Subsequently, Cell Counting Kit-8 (CCK-8), colony formation and 5-ethynyl-2'-deoxyuridine (EdU) assays were performed to analyze the influence of microRNA-548c-5p on biological functions of BCa cells. Finally, the interaction between microRNA-548c-5p and Wnt/β-catenin signaling pathway was investigated.

RESULTS: QRT-PCR results showed that the expression level of microRNA-548c-5p in BCa tumor tissues was remarkably lower than that of adjacent tissues, and the difference was statistically significant ($p<0.05$). Compared with patients with high expression of microRNA-548c-5p, the pathological stage of patients with low microRNA-548c-5p expression was significantly higher ($p<0.05$). Similarly, microRNA-548c-5p overexpression remarkably decreased the proliferation ability of BCa cells *in vitro*. However, microRNA-548c-5p knockdown showed an opposite trend. In addition, Wnt1, a key factor in the Wnt/β-catenin signaling pathway, was found remarkably up-regulated in BCa cell lines and tissues. Wnt1 expression was negatively correlated with microRNA-548c-5p expression. Western Blotting results demonstrated that microRNA-548c-5p mimics remarkably down-regulated the levels of the proteins in the Wnt/β-catenin signaling pathway. Conversely, opposite results were observed in microRNA-548c-5p inhibitor group. The rescue experiments in the cells revealed that there might be a mutual regulation between microRNA-548c-5p and Wnt1, thereby together regulating the malignant growth of BCa.

CONCLUSIONS: MicroRNA-548c-5p was lowly expressed in BCa tissues and cells, which was closely related to the pathological stage of BCa. In addition, microRNA-548c-5p significantly inhibited the proliferation of BCa cells via modulating Wnt/β-catenin signaling pathway.

Key Words:
MicroRNA-548c-5p, Wnt/β-catenin signaling pathway, Breast cancer (BCa), Proliferation.

Introduction

Breast cancer (BCa) is the most common malignant tumor in women, accounting for 7%-10% of all kinds of human malignant tumors. The incidence of BCa has increased in recent years[1,2]. Currently, the treatment of breast cancer is still based on surgery. With the improvement of early diagnosis of BCa, in addition to surgery, radiotherapy, chemotherapy, endocrine therapy and targeted therapy have also attracted more attention. Meanwhile, the survival rate of patients has greatly improved[3-5]. However, some patients still have malignant progression after treatment, such as recurrence and metastasis. This may eventually lead to cancer-related death[6,7]. Currently, several related molecular markers have been found at the molecular level. However, the specific mechanism of BCa remains unexplained[8,9]. Therefore, it is still of great clinical significance to investigate

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the mechanism of the occurrence and development of BCa at the molecular level. Furthermore, it is also vital to search for new therapeutic targets and to fundamentally improve the cure rate of BCa patients[10,11].

MicroRNAs (miRNAs) are a class of endogenous, short non-coding RNAs. They can regulate gene expression through complementary pairing with the 3′-Untranslated region (3′-UTR) bases of target gene mRNAs. This can lead to degradation or repress the translation of the target mRNAs[12-15]. MiRNA plays an important role in cell proliferation, morphology, apoptosis, and differentiation, with the characteristics of the tissue specificity. In addition, it is widely involved in the evolution of tumors[16]. Abnormal expression of miRNA regulates the biological behaviors of tumor cells by regulating the expression of the target genes[17]. Therefore, the search for new molecular targets is of great significance for the diagnosis and treatment of BCa patients, as well as the improvement of prognosis[16,17]. MicroRNA-548c-5p has been discovered for a long time. However, its biological function in malignant tumors has not been fully elucidated[18-20]. Current studies have shown that the expression of microRNA-548c-5p is down-regulated in many tumors. Acting as a tumor suppressor gene, microRNA-548c-5p participates in many physiological and pathological processes[21].

The core of research on miRNA function is to clarify how miRNA is involved in the regulation of cellular life activities by regulating target genes[21]. Therefore, the focus and difficulty of miRNA research have always been the identification and clarification of target genes and the signaling pathways regulated by miRNAs[15,17]. In recent years, no reports have focused on the regulatory effect of microRNA-548c-5p on Wnt/β-catenin signaling pathway in BCa. Therefore, the aim of this work was to investigate the roles of microRNA-548c-5p and Wnt/β-catenin signaling pathway in the occurrence and development of BCa. Our findings might help to bring new ideas for the diagnosis and treatment of BCa patients.

Patients and Methods

Patients and BCa Samples

BCa tissues and para-cancerous tissues were collected by 40 patients who underwent a radical mastectomy. No patient received radiotherapy or chemotherapy before surgery. The pathological classification and staging criteria for BCa were performed in accordance with the Union for International Cancer Control's (UICC) breast cancer staging criteria. The informed consent was obtained from patients and their families before the study. This investigation was approved by the Ethics Committee of Baoan Central Hospital of Shenzhen.

Cell Lines and Reagents

The human breast cancer cell lines (MCF-7, MDA-MB-435S, MDA-MB-231, and SKBR3) and normal mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were all purchased from Life Technologies (Gaithersburg, MD, USA). BCa cell lines were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C incubator with 5% CO_2.

Cell Transfection

MicroRNA-548c-5p mimics and microRNA-548c-5p inhibitor were purchased from Shanghai Jima Company (Shanghai, China). The cells were first seeded into 6-well plates and grown to a density of 70%. Cell transfection was performed according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours after transfection, the cells were collected for quantitative Real Time Polymerase Chain reaction (qRT-PCR) analysis and functional experiments.

Cell Counting Kit-8 (CCK-8) Assay

48 h after transfection, the cells were harvested and seeded into 96-well plates at a density of 2000 cells per well. After culture for 24 h, 48 h, 72 h, and 96 h respectively, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well, followed by incubation for 2 hours in the dark. Optical density (OD) value of each well at the absorption wavelength of 490 nm was measured by a microplate reader.

Colony Formation Assay

After 48 h of transfection, the cells were first collected. 200 cells were seeded into each well of 6-well plates, followed by culture in complete medium for 2 weeks. The medium was changed after one week, and then twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After

2 weeks, the formed colonies were washed twice with Phosphate-Buffered Saline (PBS) and fixed with 2 mL of methanol for 20 minutes. Then, the cells were stained with 0.1% crystal violet staining solution for 20 minutes, followed by washing 3 times again with PBS. The formed colonies were photographed under a light-selective environment, and the number of colonies was counted.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

To demonstrate the effect of microRNA-548c-5p on the proliferation of BCa cells, EDU proliferation assay was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μm EDU for 2 h and stained with Ado-Lo and 4',6-diamidino-2-phenylindole (DAPI). The number of EDU-positive cells was detected by fluorescence microscopy. The display rate of EDU positive was calculated as the ratio of the number of EDU positive cells to the number of total DAPI chromogenic cells (blue cells).

QRT-PCR

Total RNA in tissues and cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reverse transcribed into cDNA using the Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan) reverse transcription kit. The primers were designed using Primer 5.0 software. QRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicates were set for each sample. The Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data. Specific qRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. β-actin and U6 genes were used as internal parameters for mRNA and miRNA, respectively. The gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: Wnt1, F: 5'-CCAGCAATTATCATAATC-3', R: 5'-GCTAGCCATAGTAGACGCG-3'; microRNA-548c-5p, F: 5'-GTCCGTACAACTCCTCACATG-3', R: 5'-AGTTGGTGTATGGACGTCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. The concentration of the total protein was determined by the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride membranes (Roche, Basel, Switzerland). The Western blot analysis was performed according to standard procedures. The primary antibodies were Wnt1, β-catenin, c-myc, cyclin D1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Meanwhile, the corresponding secondary antibodies included anti-mouse and anti-rabbit. All antibodies used were purchased from Cell Signaling Technology (Danvers, MA, USA). The immunoreactive bands were finally visualized by the enhanced chemiluminescence (ECL) method.

Statistically Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. The *t*-test was used to compare the measurement data, and the categorical variables were analyzed by the χ^2-test or Fisher's exact probability method. The survival analysis was performed using the Kaplan-Meier method, and the survival curves were plotted. The experimental data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

Low Expression of MicroRNA-548c-5p in BCa Tissues and Cell Lines

The expression level of microRNA-548c-5p was remarkably reduced in BCa tissues when compared with para-cancerous tissues, and the difference was statistically significant ($p<0.05$, Figure 1A). Meanwhile, microRNA-548c-5p expression was significantly down-regulated in BCa cell lines compared with normal breast cell line ($p<0.05$). MCF-7 and MDA-MB-231 cell lines expressed the lowest level of microRNA-548c-5p, which were selected for subsequent experiments (Figure 1B). QRT-PCR results showed that microRNA-548c-5p was lowly expressed in BCa tissues and cell lines.

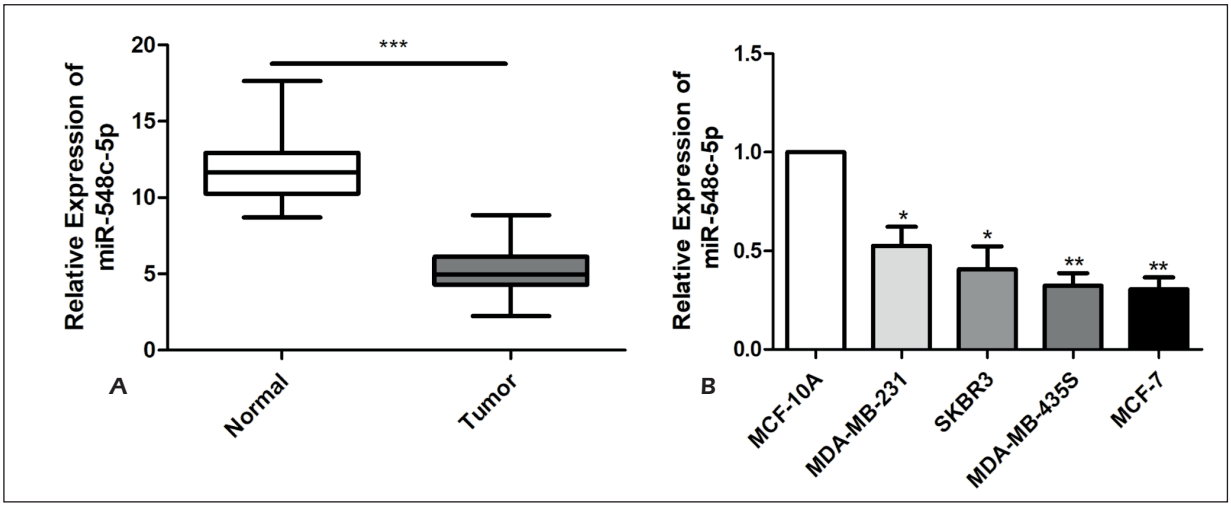

Figure 1. MiR-548c-5p was lowly expressed in BCa tissues and cell lines. **A**, QRT-PCR was used to detect the expression of miR-548c-5p in BCa tissues and adjacent tissues. **B**, QRT-PCR was used to detect the expression level of miR-548c-5p in BCa cell lines. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

MicroRNA-548c-5p Expression Was Correlated With Pathological Stage of BCa Patients

According to the expression of microRNA-548c-5p, 40 pairs of BCa tissues were divided into high microRNA-548c-5p expression group and low microRNA-548c-5p expression group. The relationship between microRNA-548c-5p expression with age, pathological stage, lymph node metastasis, and distant metastasis of BCa patients was analyzed. As shown in Table I, the low expression of microRNA-548c-5p was positively correlated with the pathological stage of BCa, whereas it was not associated with age, lymph node metastasis, and distant metastasis. All these results indicated that microRNA-548c-5p expression was correlated with the pathological stage of BCa patients.

Overexpression/Knockdown of MicroRNA-548c-5p Inhibited/ Promoted BCa Cell Proliferation

To explore the effect of microRNA-548c-5p on cytology of BCa, microRNA-548c-5p mimics, and microRNA-548c-5p inhibitor were transfected into MCF-7 and MDA-MB-231 cell lines, respectively. QRT-PCR results verified the transfection efficiency (Figure 2A). Subsequent CCK-8 assay showed that the proliferation ability of cells in microRNA-548c-5p mimics group significantly de-

Table I. Association of miR-548c-5p expression with clinicopathologic characteristics of breast cancer.

Parameters	Number of cases	MiR-548c-5p expression		*p*-value*
		High (%)	Low (%)	
Age (years)				0.945
<60	14	9	5	
≥60	26	17	9	
T stage				0.010
T1-T2	25	20	5	
T3-T4	15	6	9	
Lymph node metastasis				0.305
No	27	19	8	
Yes	13	7	6	
Distance metastasis				0.251
No	30	21	9	
Yes	10	5	5	

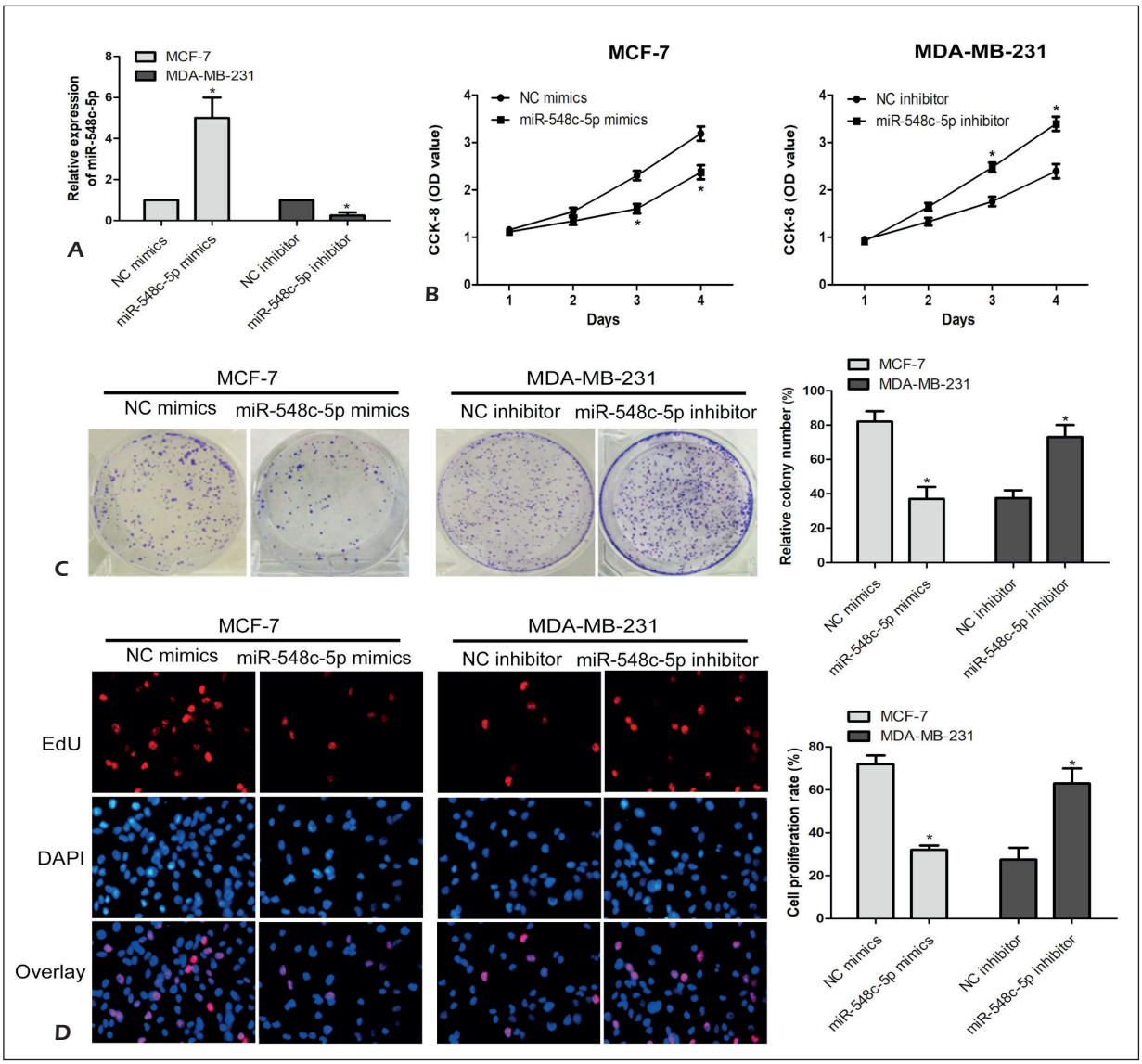

Figure 2. Overexpression/silencing of miR-548c-5p inhibited/promoted BCa cell proliferation. **A**, QRT-PCR verified the interference efficiency after the transfection of miR-548c-5p inhibitor in MCF-7 cell line and miR-548c-5p mimics in MDA-MB-231 cell line. **B**, CCK-8 assay detected the effects of miR-548c-5p transfection on BCA cell proliferation. **C**, Colony formation assay was performed to detect the number of clones formed by MCF-7 and MDA-MB-231 cell lines (magnification × 20). **D**, EdU assay detected positive proliferating MCF-7 and MDA-MB-231 cells (magnification × 40). Data were expressed as mean ± SD, *$p<0.05$.

creased ($p<0.05$, Figure 2B). However, the proliferation ability of the cells in microRNA-548c-5p inhibitor group remarkably increased ($p<0.05$). In addition, the colony formation and EdU results also showed that the number of BCa cells in microRNA-548c-5p mimics group was remarkably reduced when compared with the NC group ($p<0.05$). This suggested that the proliferative capacity was inhibited after transfection of microRNA-548c-5p mimics. Conversely, opposite results were observed after transfection of microRNA-548c-5p inhibitor (Figures 2C-2D). These results demonstrated that microRNA-548c-5p could modulate the proliferation of BCa cells.

High Expression of Wnt1 in BCa Tissues and Cell Lines

Later, we explored the underlying mechanism of microRNA-548c-5p in BCa. Bioinformatics predicted that there might be some association between microRNA-548c-5p and Wnt1 in BCa. QRT-PCR results demonstrated that the expression of Wnt1 was remarkably upregulated in BCa tumor tissues compared with para-cancerous tis-

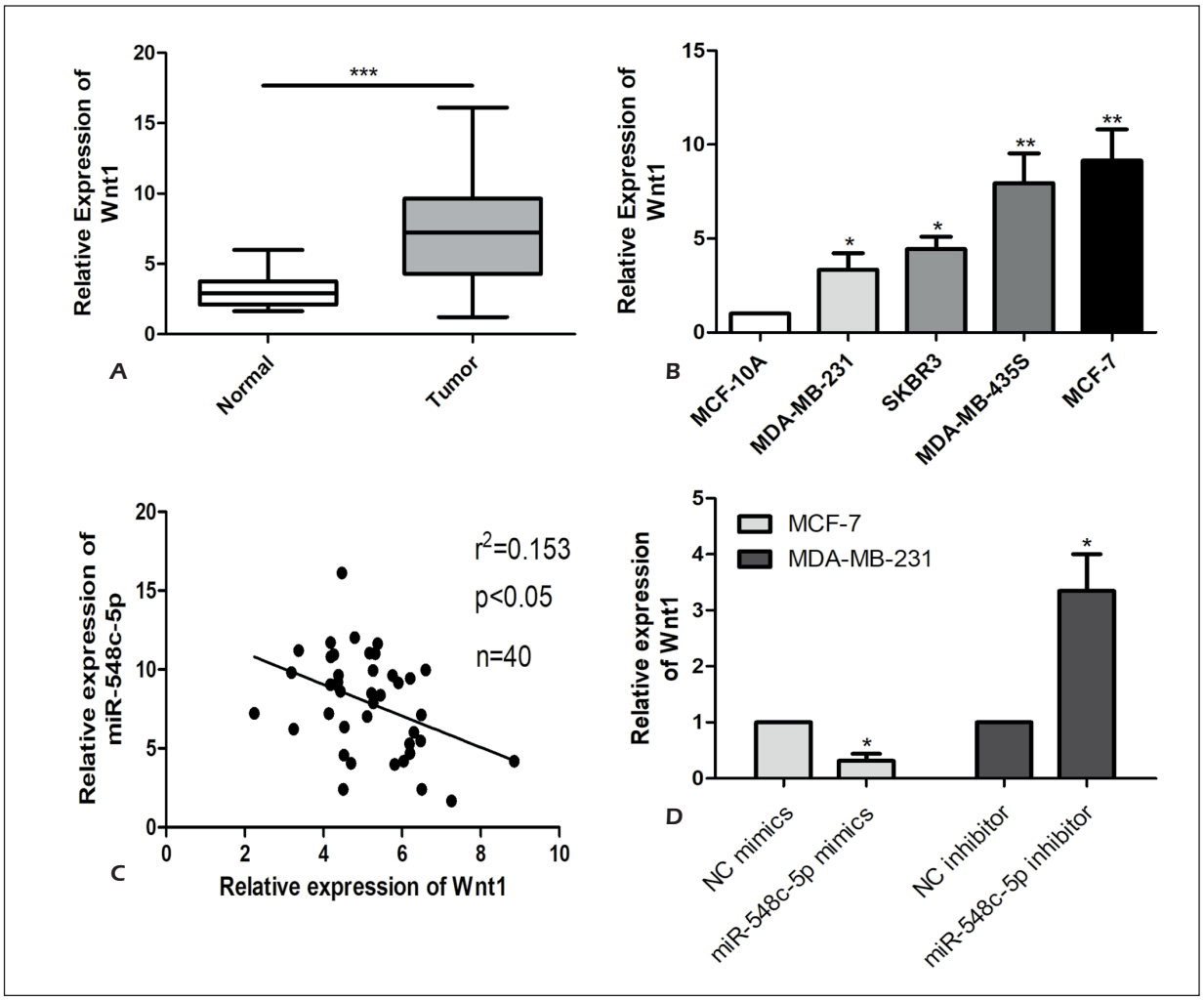

Figure 3. High expression of Wnt1 in BCa tissues and cell lines. **A**, QRT-PCR was used to detect Wnt1 expression in BCa tissues and adjacent tissues. **B**, QRT-PCR was used to detect the expression level of Wnt1 in BCa cell lines. **C**, There was a significant negative correlation between miR-548c-5p and Wnt1 expression in BCa tissues. **D**, The expression level of Wnt1 after transfection of miR-548c-5p was detected by qRT-PCR. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

sues (p<0.05, Figure 3A). In addition, Wnt1 expression was remarkably higher in BCa cell line than MCF-10A cells, and the difference was statistically significant (p<0.05, Figure 3B). Next, we detected the expressions of microRNA-548c-5p and Wnt1 in BCa tissues and adjacent tissues by qRT-PCR. The results indicated that the expression level of microRNA-548c-5p was negatively correlated with Wnt1 in BCa tissues (Figure 3C). Furthermore, the Wnt1 expression level significantly decreased in microRNA-548c-5p mimics group compared with the NC group, while Wnt1 expression remarkably increased in microRNA-548c-5p inhibitor group (p<0.05, Figure 3D).

Over-Expression/Knockdown of MicroRNA-548c-5p Changed Wnt/β-Catenin Signaling Pathway in BCa Cell Lines

Western Blotting results showed that the expression levels of the proteins in Wnt/β-catenin signaling pathway (including Wnt1, β-catenin, c-myc, and cyclin D1) were remarkably down-regulated in microRNA-548c-5p mimics group when compared with NC group (p<0.05). However, microRNA-548c-5p knockdown remarkably decreased the expressions of Wnt/β-catenin signaling pathway proteins (p<0.05, Figure 4).

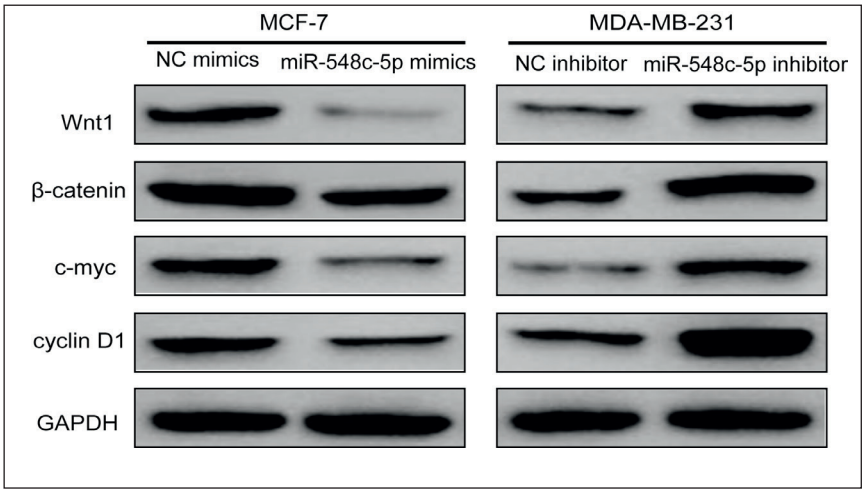

Figure 4. Overexpression/silencing of miR-548c-5p down-regulated/upregulated the expression of the Wnt/β-catenin signaling pathway. The Western Blot verified the expression level of Wnt/β-catenin signaling pathway after transfection of miR-548c-5p in MCF-7 and MDA-MB-231 cell lines.

MicroRNA-548c-5p Modulated Wnt1 in Human BCa Cells

To further explore the mechanism in which microRNA-548c-5p and Wnt1 inhibited the malignant progression of BCa, we overexpressed Wnt1 in MCF-7 cells transfected with microRNA-548c-5p mimics, or silenced Wnt1 in MDA-MB-231 cells transfected with microRNA-548c-5p inhibitor. The transfection efficiency was examined by qRT-PCR and Western Blotting (Figure 5A). The subsequent colony formation assay demonstrated that the knockdown of Wnt1 remarkably increased the number of BCa cells in microRNA-548c-5p inhibitor group, thereby counteracting the effect of microRNA-548c-5p on the inhibition of BCa cell proliferation (Figure 5B).

Discussion

With the progress of the Human Genome Project, we entered the era of molecular understanding[8,9]. Since the discovery and the in-depth study of miRNAs, it has been recognized that miRNAs are widely involved in cell differentiation, proliferation, apoptosis, migration, and invasion[10,11]. Current researches have found that the abnormal expression of miRNAs is related to tumor development to some extent. Meanwhile, investigations[12,13] have paid more and more attention to the roles of miRNAs in tumor progression. Many studies have shown that miRNAs exert important effects on the growth, invasion, and apoptosis of human tumors. In addition, the dysregulation of miRNAs plays an important role in the growth, invasion, and apoptosis of tumor cells[14-16].

Current studies on the relationship between miRNA and BCa have mainly focused on the following three points: 1) to improve the expression profile of miRNA in BCa tissues; 2) to study the expression and function of miRNAs related to BCa; 3) to study the application value of miRNAs in BCa occurrence, development, diagnosis, and treatment and prognosis. MiRNAs exhibit specific expression patterns between normal tissues and tumor tissues, as well as in tumor tissues with different degrees of differentiation. Using miRNAs as potential targets, researchers have found that more than half of miRNAs are located at "fragile sites" on chromosomes. Some miRNAs have been found located at the relevant breaking point area of human BCa. This may be the reason for the relationship between tumor occurrence and the abnormal expression of specific miRNAs[16,17]. A large number of reports[14,16] have confirmed that the expression of some miRNAs is positively or negatively correlated with malignant tumors. These findings suggest that miRNA plays an important role in the occurrence and development of tumors. Furthermore, miRNAs act as oncogenes or tumor suppressor genes in different tumors through the regulation of the target genes[17].

Although microRNA-548c-5p has been discovered for a long time, its biological function has not been fully elucidated. MicroRNA-548c-5p plays a role in tumor inhibition. It is also involved in many physiological and pathological processes of malignancies, such as hepatocellular carcinoma, colorectal cancer, etc. Many researches have suggested that microRNA-548c-5p plays a pivotal role in the development of BCa[18-20]. However, the specific role of microRNA-548c-5p in BCa is unknown. To further explore the exact role of microRNA-548c-5p in the occurrence and development of BCA, qRT-PCR was applied to detect the expression of microRNA-548c-5p in 40 BCa tissues and para-cancer

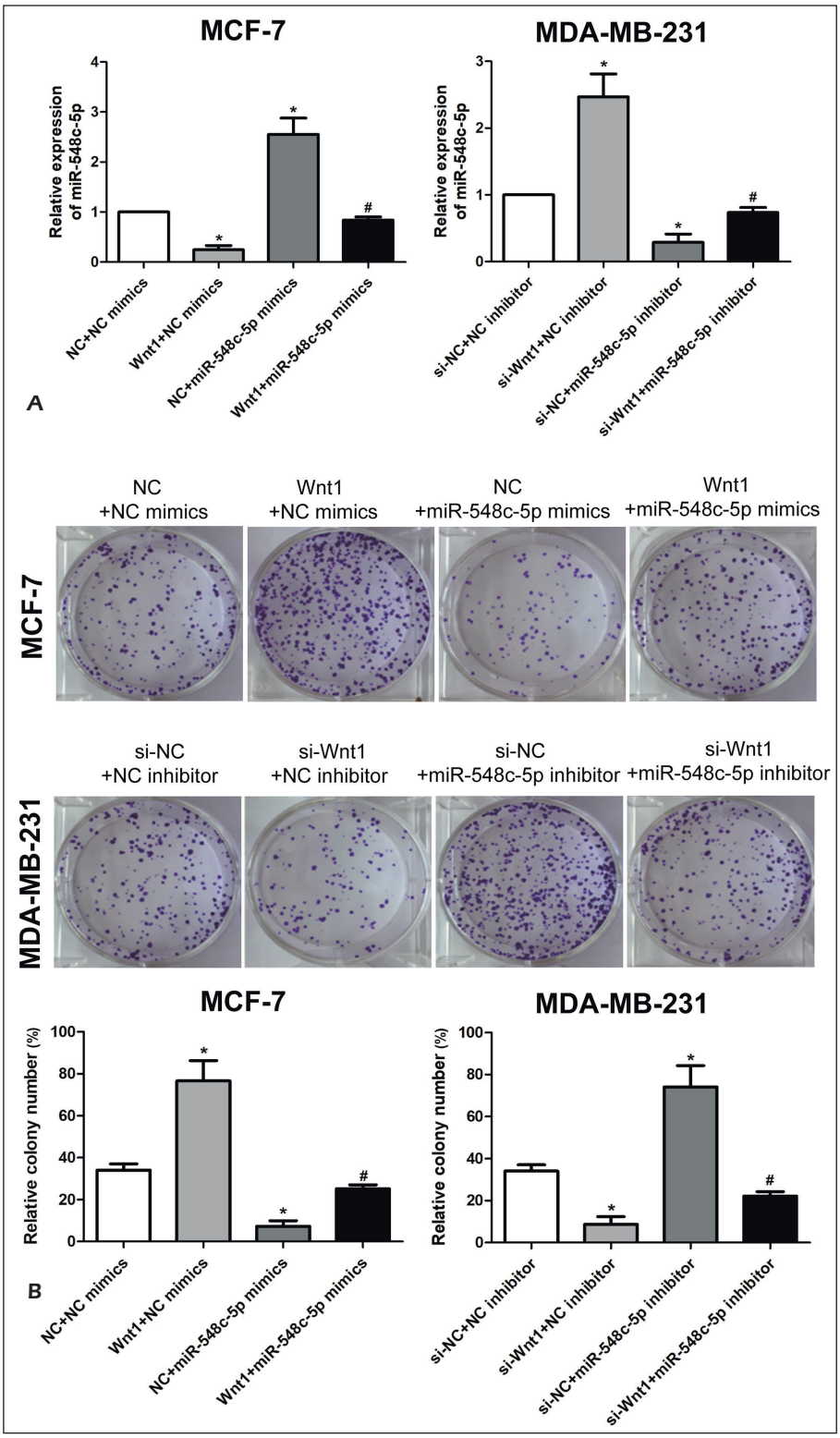

Figure 5. MiR-548c-5p regulated the expression of Wnt1 in BCa tissues and cell lines in the Wnt/β-catenin signaling pathway. **A**, The expression level of miR-548c-5p in BCa cell lines co-transfected with miR-548c-5p and Wnt1 was detected by qRT-PCR. **B**, Colony formation assay detected the number of clones in BCa cells after co-transfection of miR-548c-5p and Wnt1 (magnification × 20). Data were expressed as mean ± SD, $*^{\#}p<0.05$.

tissues in this study. The results found that the expression of microRNA-548c-5p in BCa tissues was remarkably lower than that of para-cancer tissues. This suggested that microRNA-548c-5p might act as a tumor suppressor gene in BCa. Meanwhile, the expression level of microRNA-548c-5p was found remarkably down-regulated in BCa cell lines as well. To further explore the effect of mi-

croRNA-548c-5p on the biological functions of BCa, the microRNA-548c-5p overexpression and the knockdown models were constructed. CCK-8, colony formation, and EdU assays showed that microRNA-548c-5p could significantly inhibit the proliferation of BCa. However, the specific molecular mechanism still remained elusive.

Wnt1 is the general name of a large class of Wnt genes, which can encode secreted signal transduction proteins with similar structures. However, the functions of several of these proteins are repeated[22,23]. In recent years, the major intracellular signal transduction pathways involved in the activation of secreted signal transduction proteins have been elucidated. The components of this pathway include proto-oncogene products (e.g., β-catenin) and tumor suppressor proteins (e.g., APC)[23]. It has been previously reported that Wnt1 protein itself is not involved in human BCa. However, other Wnt genes are often highly expressed in BCa. Meanwhile, the proportion of the downstream components of the Wnt pathway activated in BCa is remarkably elevated[22,23]. In fact, cyclin D1 is often highly expressed in BCa patients, which plays a major role in BCa cell proliferation[24]. Thus, the abnormal activity of the Wnt/β-catenin pathway is an important marker of BCa[22-24]. In this study, the expression of key factors in the Wnt/β-catenin signal pathway after microRNA-548c-5p transfection was detected by the Western Blotting. The results showed that the protein level of the related factors in the Wnt/β-catenin signal pathway was remarkably down-regulated by the overexpression of microRNA-548c-5p. Subsequent recovery experiment demonstrated that knockdown of Wnt/β-catenin signaling pathway remarkably increased the number of BCa cell clone formation in microRNA-548c-5p inhibitor group. This might counteract the effect of microRNA-548c-5p on BCa cell proliferation. Therefore, it was suggested that microRNA-548c-5p inhibited the malignant progression of BCa by regulating the Wnt/β-catenin signaling pathway.

Conclusions

In brief, MicroRNA-548c-5p was remarkably down-regulated in BCa and was associated with pathological staging. In addition, microRNA-548c-5p could inhibit the occurrence and development of BCa by regulating the Wnt/β-catenin signaling pathway.

Acknowledgments

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Conflict of Interests

The authors declared that they have no conflict of interests.

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