European Review for Medical and Pharmacological Sciences 2019; 23: 9489-9498

DCLK1 promotes malignant progression of breast cancer by regulating Wnt/β-Catenin signaling pathway

Y.-L. WANG[1], Y. LI[2], Y.-G. MA[3], W.-Y. WU[4]

[1]Department of Thyroid Mammary, First People's Hospital, Yangquan, China
[2]Department of Thyroid & Breast Surgery, the Fifth Affiliated Hospital, Sun Yat-Sen University, Zhuhai, China
[3]Department of Cardiothoracic Surgery, First People's Hospital, Yangquan, China
[4]Department of Oncology, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, China

Yongliang Wang and *Yi Li* contributed equally to this work

Abstract. – OBJECTIVE: The study was aimed to investigate the expression of double-cortin-like kinase-1 (DCLK1) in breast cancer (BCa) tissues and cells and further study its association with clinicopathology and prognosis of BCa patients.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to examine the expression of DCLK1 in 44 BCa tumor tissues, as well as adjacent normal tissues. Also, the interplay between DCLK1 level and clinical data or the prognosis of BCa patients was analyzed. QRT-PCR was further used to verify the level of DCLK1 in BCa cell lines. In addition, the DCLK1 knockdown model was constructed using lentivirus in BCa cell lines. Next, cell counting kit-8 (CCK-8) and cell clone formation and tranwell assays were used to analyze the effect of DCLK1 on the biological function of BCa cells. Finally, it was explored whether DCLK1 can act through the Wnt/β-Catenin signaling pathway.

RESULTS: In this research, qRT-PCR results revealed that the level of DCLK1 in BCa tumor tissues was remarkably higher than in adjacent tissues. Compared to patients with a low-level of DCLK1, the pathology grading in patients with high-level was higher and the overall survival rate was lower. Similarly, proliferation, as well as the invasion and migration ability of cells in DCLK1 knockdown group was remarkably down-regulated when compared to negative control group. Moreover, the Western Blot results revealed that silencing DCLK1 remarkably decreased the expression of key proteins in Wnt/β-Catenin pathways such as β-Catenin, c-myc, and cyclinD1, thereby promoting the malignant progression of BCa. In addition, the Wnt/β-Catenin pathway inhibitor was found to be able to reverse the impact of DCLK1 overexpression on BCa cell proliferative and metastatic capacity.

CONCLUSIONS: DCLK1 expression was found remarkably increased in BCa tissues and closely associated with the pathological stage, as well as poor prognosis of BCa patients. Furthermore, DCLK1 may promote the malignant progression of BCa by inhibiting the Wnt/β-Catenin pathway.

Key Words:
DCLK1, Wnt/β-Catenin, Breast cancer, Malignant progression.

Introduction

As one of the most common malignant tumors, breast cancer (BCa) kills about 350,000 people every year, posing a serious threat to women's life and health[1,2]. Many factors are potentially associated with the occurrence of breast cancer, including age, topical hormones, overweight, alcoholism, and smoking[3,4]. In the past decade, the diagnosis and treatment of breast cancer has developed by leaps and bounds. However, due to its high incidence and mortality, it is still a major killer of women's health[5,6]. Therefore, people pay more attention to the molecular mechanism of breast cancer, hoping to find out the mechanism of its pathogenesis and development[7]. Early diagnosis and treatment can greatly reduce the further occurrence of tumor deterioration. Recently, more efforts have been paid off by researchers to

Corresponding Author: Wenyi Wu, MM; e-mail: Wwyii8522858@163.com

explore the potential molecular markers of recurrence and metastasis, which may provide a new guidance for the treatment of breast cancer[8,9].

Doublecortin-like kinase-1 (DCLK1) is a member of the protein kinase superfamily and the doublecortin family, whose coding gene is located on human chromosome 13q13.3[10,11]. Some studies[12,13] have demonstrated that DCLK1 is expressed in the cytoplasm and matrix of the epithelial cells of most malignant tumor tissues such as breast cancer, pancreatic cancer, and prostate cancer, and is associated with tumor metastasis and poor prognosis. Further studies[13,14] have found that DCLK1 has cancer stem cell-like characteristics in breast cancer cell lines, indicating that DCLKI has the potential as an early diagnostic indicator for breast cancer and is valuable for tumor recurrence and disease prognosis. Although many tumor suppressor genes and oncogenes (such as APC, KRAS, and P53) have been found in breast cancer, many low-frequency somatic mutations lead to the heterogeneity of breast cancer[15]. Due to the increasing number of potential functional mutations, none of the treatments for breast cancer are completely curable[16].

Wnt is a kind of secreted glycoprotein widely existing, which has a large molecular weight and plays a certain role as a messenger and is related to the transmission of cell signals to a certain extent[17,18]. However, when the external factors change, this glycoprotein is overactivated or maladjusted, which is likely to cause abnormal cell proliferation and form tumors[19,20]. At present, the mechanism of action of glycoprotein in the tumor is still not clear, and there are few reports on the related research[20]. Although the APC gene has been detected in a variety of tumors, its chances of mutation are lower. The β-Catenin gene is relatively more likely to be mutated[21]. In gastric cancer without APC gene mutation, the phosphorylation process of this gene is hindered after β-Catenin gene mutation, leading to its massive aggregation[22,23]. Mutations of this gene have been found in many tumors, indicating a close relation between mutations of this gene and tumors[24]. Based on the above facts, it can be inferred that any genetic mutation leading to increased levels of β-catenin in cells can cause precancerous lesions or tumors; therefore, we predict whether DCLK1 can be involved in the process of breast cancer cell proliferation and metastasis through the targeted regulation of the Wnt/β-catenin signal pathway.

Patients and Methods

Patients and BCa Samples

44 specimens of breast cancer tissue and adjacent tissues were obtained from fresh specimens of biopsy or surgical resection from BCa patients in our hospital and stored in a refrigerator at –80°C. The 44 patients with breast cancer were aged from 30 to 78 years, with an average of 64.9 (56.9-79.8) years. All cases were diagnosed by two senior directors of pathology to confirm the results accurately. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

The human breast cancer cell lines (MCF-7, MDA-MB-231, and SKBR3) and normal mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Both Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL), and placed in an incubator with 5% CO_2 at 37°C. The cells were passaged with 1×trypsin+EDTA (ethylenediaminetetraacetic acid) when the cell density reached 80-90%.

Cell Transfection

The control group (NC) and DCLK1 (DCLK1-S) containing the DCLK1 lentiviral sequence were purchased from the Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 70%, lentiviral transfection was performed according to the manufacturer's instructions, and cells were collected 48 h later for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Western Blot analysis, and cell function experiments.

Cell Proliferation Assay

The proliferation of the three cell lines was examined using the Cell Counting Kit-8 (CCK-8) kit (Dojindo, Kumamoto, Japan). The main steps were as follows. First, 100 μL of cell suspension (containing 2000 cells) was added per well. Then, 10 μL of CCK-8 solution was added to incubate

the cells for 1 h in a culture incubator. Lastly, the optical density (OD) value of each well was measured using a microplate reader at 450 nm. Wells containing the corresponding amount of cell culture medium and CCK-8 solution but no cells were considered as a blank control.

Colony Formation Assay

The cells were collected after 48 h of transfection and 200 cells were seeded in each well of a 6-well plate, and cultured in complete medium. After 2 weeks, the cells were cloned, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Once cells were fixed in 2 mL of methanol for 20 min, methanol was aspirated and cells were stained with 0.1% crystal violet staining solution for 20 min. Then, once washed 3 times with PBS, cells were photographed and counted under a light-selective environment.

Transwell Assay

The cells after transfection for 48 h were digested, centrifuged, and resuspended in a medium without FBS to adjust the density to $5x10^5$ cells/mL. 200 μL of cell suspension (1x105 cells) was added to the upper chamber, and 700 μL of a medium containing 20% FBS was added to the lower chamber. According to the different migration abilities of each cell line, cells were put back into the incubator and continued to culture for a specific time. The transwell chamber was taken out, washed 3 times with 1xPBS, and placed in methanol for cell fixation for 15 min. 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 water and a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under a microscope, and 5 fields of view were randomly selected.

QRT-PCR

Total RNA was extracted from BCa cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR reaction was carried out using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga Japan), and StepOne Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: DCLK1: forward: 5'-TAGCCAGGGCCATCAAATAC-3', reverse: 5'-ACCCAGCTTCAGTGATTTGC-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using the ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Transfected cells were lysed using the cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 x g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies were DCLK1, β-Catenin, c-myc, cyclinD1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary antibodies were anti-mouse and anti-rabbit, all purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using the One-way ANOVA test followed by Post-Hoc test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

DCLK1 Was Highly Expressed in BCa Tissues and Cell Lines

To determine the level of DCLK1 in BCa tissues, we collected 44 pairs of BCa tumor tissues and adjacent non-tumor tissues and detected the differential level by qRT-PCR. Results showed that DCLK1 expression was elevated in BCA tissues compared with the normal ones (Figure 1A),

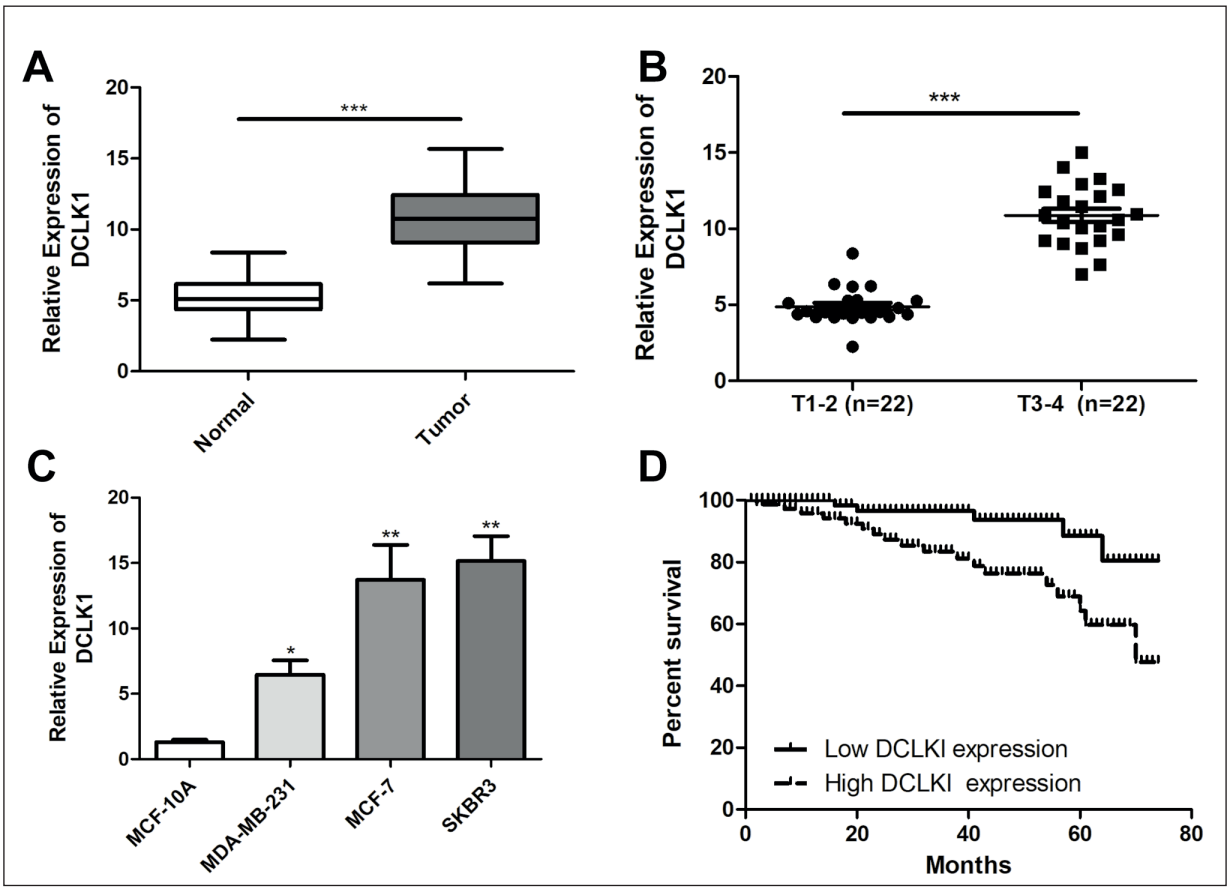

Figure 1. DCLK1 was highly expressed in breast cancer tissues and cell lines. **A,** qRT-PCR was used to detect the differential expression of DCLK1 in breast cancer tissues and adjacent tissues. **B,** qRT-PCR was used to detect the differential expression of DCLK1 between the histopathological grades of breast cancer tumors T1-2 and T3-4. **C,** qRT-PCR was used to detect the expression level of DCLK1 in breast cancer cell lines. **D,** Kaplan-Meier survival curve of breast cancer patients based on DCLK1 expression; the prognosis of patients with high expression was significantly worse than that of low expression group. Data are mean ± SD, *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

suggesting that DCLK1 may act as a tumor-promoting gene in BCa. In addition, we found that the DCLK1 level was higher in patients in BCa T3-4 stage than in patients in the T1-2 stage (Figure 1B). At the same time, DCLK1 level in BCa cell lines was also found remarkably higher than in MCF-10A, the normal breast tissue cell line (Figure 1C).

DCLK1 Level Was Correlated with Clinical Stage and Overall Survival in BCa Patients

According to the qRT-PCR results of 44 pairs of DCLK1 level in BCa tissues and adjacent tissues, DCLK1 level was divided into high-level group and low-level group, and the number of each group was counted. Chi-square test was used to analyze the interplay between DCLK1 level and age, gender, pathological stage, lymph node metastasis, and distant metastasis of BCa patients. As shown in Table I, highly-expressed DCLK1 is positively correlated with the pathological stage of BCa. In addition, to further explore the association between DCLK1 expression and the prognosis of BCa patients, we collected relevant follow-up data. The Kaplan-Meier survival curves revealed that high expression of DCLK1 was remarkably associated with poor prognosis of BCa patients. The higher the DCLK1 level, the worse the prognosis ($p<0.05$; Figure 1D).

Knockdown of DCLK1 Inhibited Cell Proliferation in BCa Cells

To explore the effect of DCLK1 on the proliferation of BCa cells, we first constructed

Table. Association of DCLK1 expression with clinicopathologic characteristics of breast cancer.

Parameters	Number of cases	DCLK1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.697
< 60	20	12	8	
≥ 60	24	13	11	
Gender				0.759
Male	15	9	6	
Female	29	16	13	
T stage				0.033
T1-T2	22	16	6	
T3-T4	22	9	13	
Lymph node metastasis				0.086
No	18	13	5	
Yes	26	12	14	
Distance metastasis				0.186
No	28	18	10	
Yes	16	7	9	

a DCLK1 interference model and verified its transfection efficiency by qRT-PCR and Western blot (Figures 2A and 2B). Subsequently, CCK8 and cell clone formation assays were used to detect cell proliferation of control group and DCLK1 interference group (DCLK1-S). As a result, it was found that the cell proliferation rate of the DCLK1-S group was remarkably decreased when compared with NC group (Figures 2C and 2D).

Knockdown of DCLK1 Inhibited BCa Cell Invasion and Migration

To explore the effect of DCLK1 on the ability of BCa cells to metastasize, we performed transwell assay to detect the cell invasion and migration abilities of NC and DCLK1-S group. Results indicated that the rate of invasion and migration of cells in the DCLK1-S group was remarkably lower than in NC group (Figure 2E).

Knockdown of DCLK1 Decreased the Expression of Wnt/β-Catenin Pathway

To further explore the ways in which DCLK1 promotes the malignant progression of breast cancer, we performed Western Blot assay to examine the levels of key proteins including β-Catenin, c-myc, and cyclinD1 in the Wnt/β-Catenin pathway, founding that they were remarkably decreased after silencing DCLK1 (Figure 3).

Wnt/β-Catenin Pathway Inhibitor Reversed DCLK1 Induced Carcinogenesis

To further explore the impact of DCLK1 on the proliferation of BCa cells, we constructed DCLK1 overexpression model and added the Wnt/β-Catenin pathway inhibitor (Wnt/β-Catenin-IN-1), and the DCLK1 mRNA and protein expression were analyzed by qRT-PCR and Western blot (Figures 4A and 4B). Subsequently, CCK8 and cell clone formation assay were used to detect the cell proliferation of DCLK1 and DCLK1+Wnt/β-Catenin-IN-1 group. As a result, the cell proliferation rate of the DCLK1+Wnt/β-Catenin-IN-1 group was found remarkably decreased as compared with DCLK1 group (Figures 4C and 4D). In addition, the results of flow cytometry showed that the cell apoptosis rate of DCLK1+Wnt/β-Catenin-IN-1 group was remarkably higher than that of DCLK1 group (Figure 4E).

Discussion

Malignant tumor is the first lethal factor threatening human life[1,2]. According to the latest data survey, 12.7 million new malignant tumors were diagnosed in the world in the latest year, among which 7.6 million patients died[1-3]. There is no doubt that breast cancer is the leading cause of death among women[1,4]. Mutations in normal cells occur in the progression of tumor cells, including morphological changes, growth, loss of contact, inhibition, hyperproliferation, invasion, and migration, as well as increased expression of

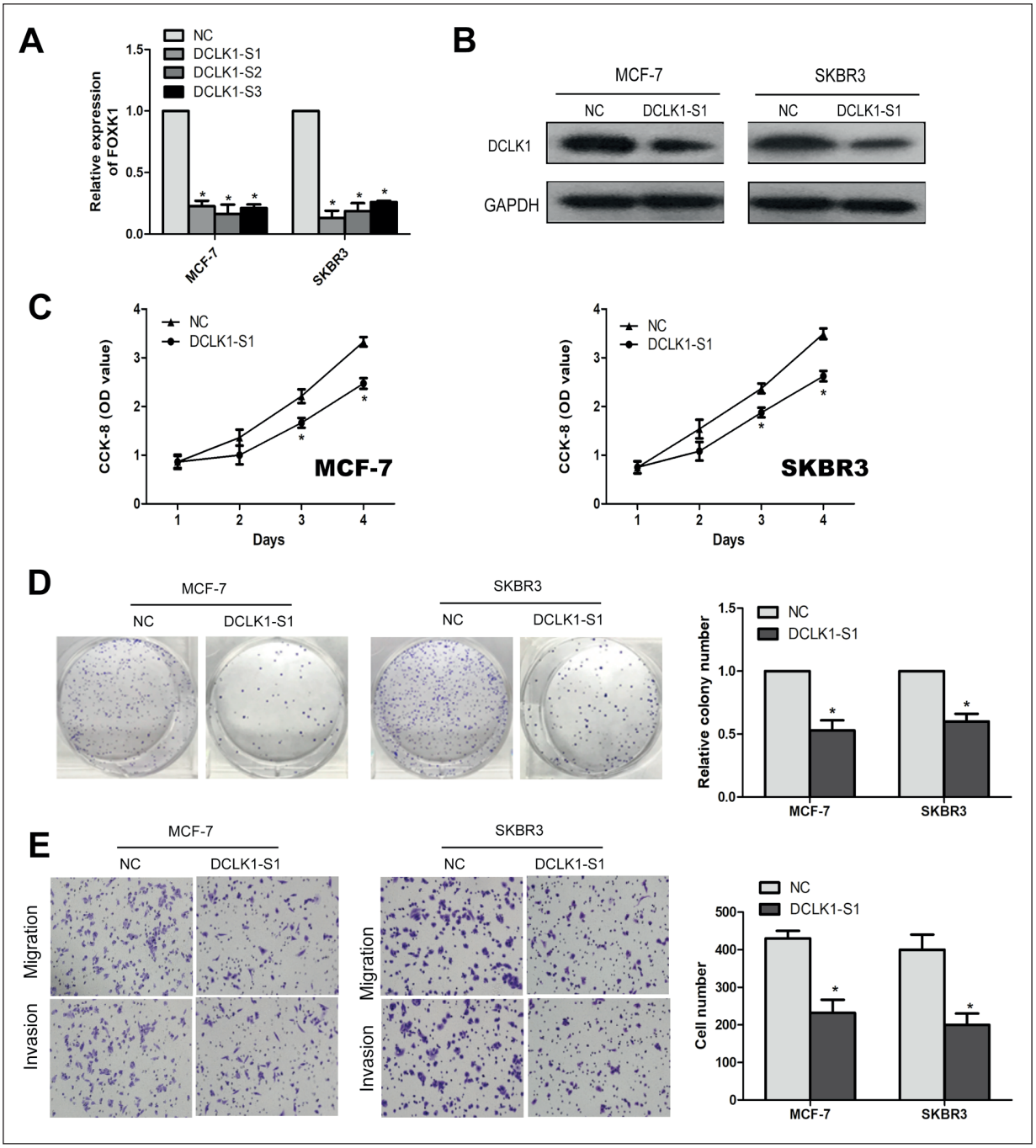

Figure 2. Inhibition of breast cancer cell proliferation and metastasis after silencing DCLK1. **A,** qRT-PCR verified the interference efficiency after small interference transfection of DCLK1 in MCF-7 and SKBR3 cell lines. **B,** Western blot verified the interference efficiency after transfection of DCLK1 in MCF-7 and SKBR3 cell lines. **C,** CCK-8 assay detected the effect of DCLK1 interference on MCF-7 and SKBR3 cell lines. **D,** Cell cloning experiments examined the effects of DCLK1 interference on proliferation of MCF-7 and SKBR3 cell lines. **E,** Tranwell assay detected the effects of DCLK1 interference on invasion and migration of MCF-7 and SKBR3 cell lines (magnification: 40×). Data are mean ± SD, *$p<0.05$.

malignant related genes[4-6]. A series of molecular biological changes and their regulatory mechanisms in the process of cell malignant transformation and tumor progression are still unclear, further revealing the strong functions possessed by oncogenes and tumor suppressor genes, pro-

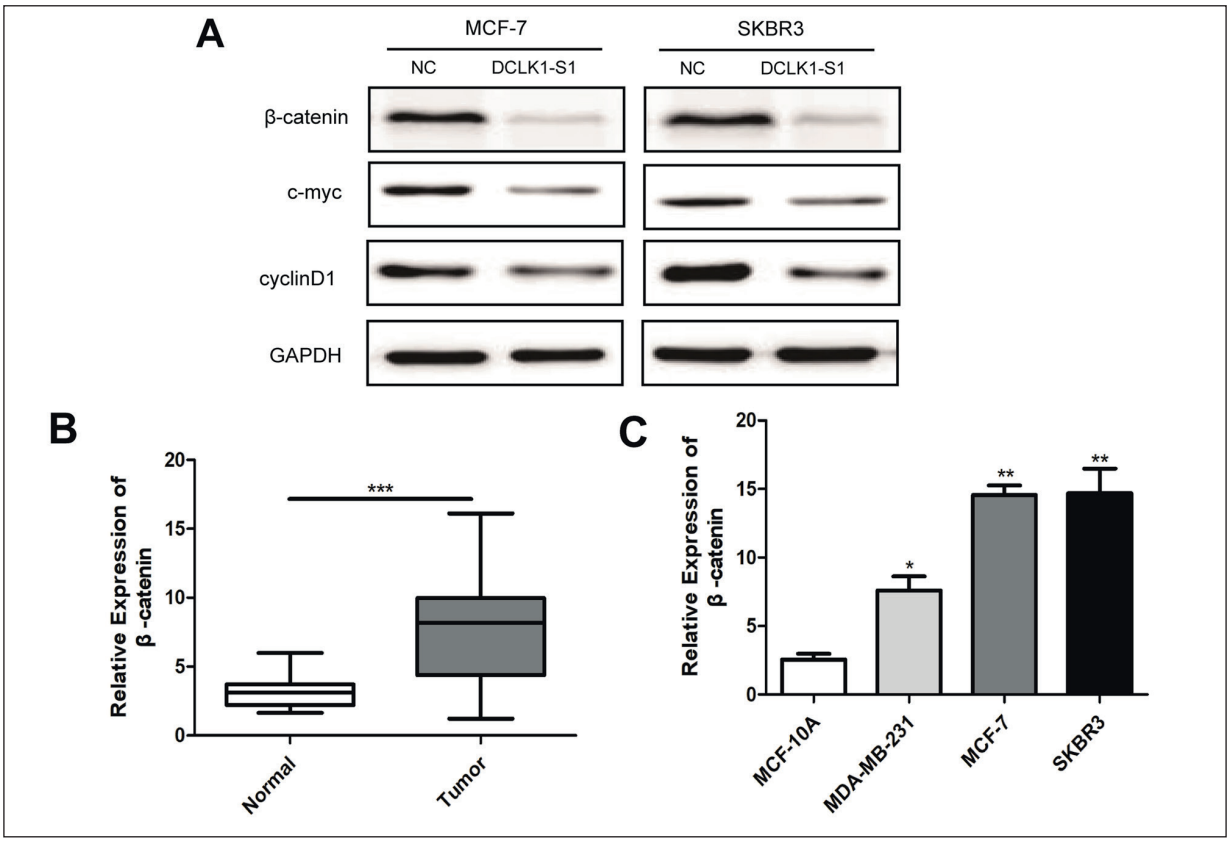

Figure 3. DCLK1 regulated the mechanism of action of Wnt/β-Catenin signaling pathway in breast cancer cells. **A,** Western blotting verified the expression levels of β-Catenin, c-myc, and cyclinD1 after interference with DCLK1 in MCF-7 and SKBR3 cell lines. **B,** qRT-PCR was used to detect the difference in expression of SMAD2 in breast cancer tissues and adjacent tissues. **C,** qRT-PCR was used to detect the expression level of SMAD2 in breast cancer cell lines. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

viding support for understanding the process of tumor development and finding new and feasible therapeutic targets[7-9].

DCLK1 gene is a transmembrane microtubule-associated protein kinase originally found in the nervous system[10-12]. The encoded protein has two N-terminal microtubule-associated protein domains (Doublecortin, DCX) and one C-terminal serine/threonine protein kinase domain[13]. The two DCX structures are DCXl and DCX2 or N-DCX and C-DCX respectively; among them, DCX1 has a typical DCX domain characteristic to specifically bind microtubules, while DCX2 mainly interacts with microtubules and their dimers[14,15]. The DCX domain can combine with microtubules in the nervous system and regulate microtubule aggregation to regulate the migration of neurons. The C-terminal is similar to calmodulin-dependent kinase but lacks a typical calmodulin-binding site[16]. Both DCLK1 and DCX are members of the DCX family, in which DCX acts as a marker for neuronal precursor cells and plays a catalytic role in regulating neuronal migration and axonal growth[14-16]. Meanwhile, through *in vivo* and *in vitro* experiments, it is found that DCX is closely related to tumor invasion, but the specific mechanism remains to be further studied.

In our study, we first applied a large number of clinical specimens of breast cancer to explore the role of DCLK1 in the development of breast cancer. We examined the expression of DCLK1 at the transcriptional and protein levels in fresh breast cancer surgical specimens and breast cancer cell lines, and found that the expression of DCLK1 was increased in most breast cancer tissues and lines in different degrees compared to adjacent tissues and normal breast cancer cell line. Therefore, the above experimental results indicated that the high expression of DCLK1

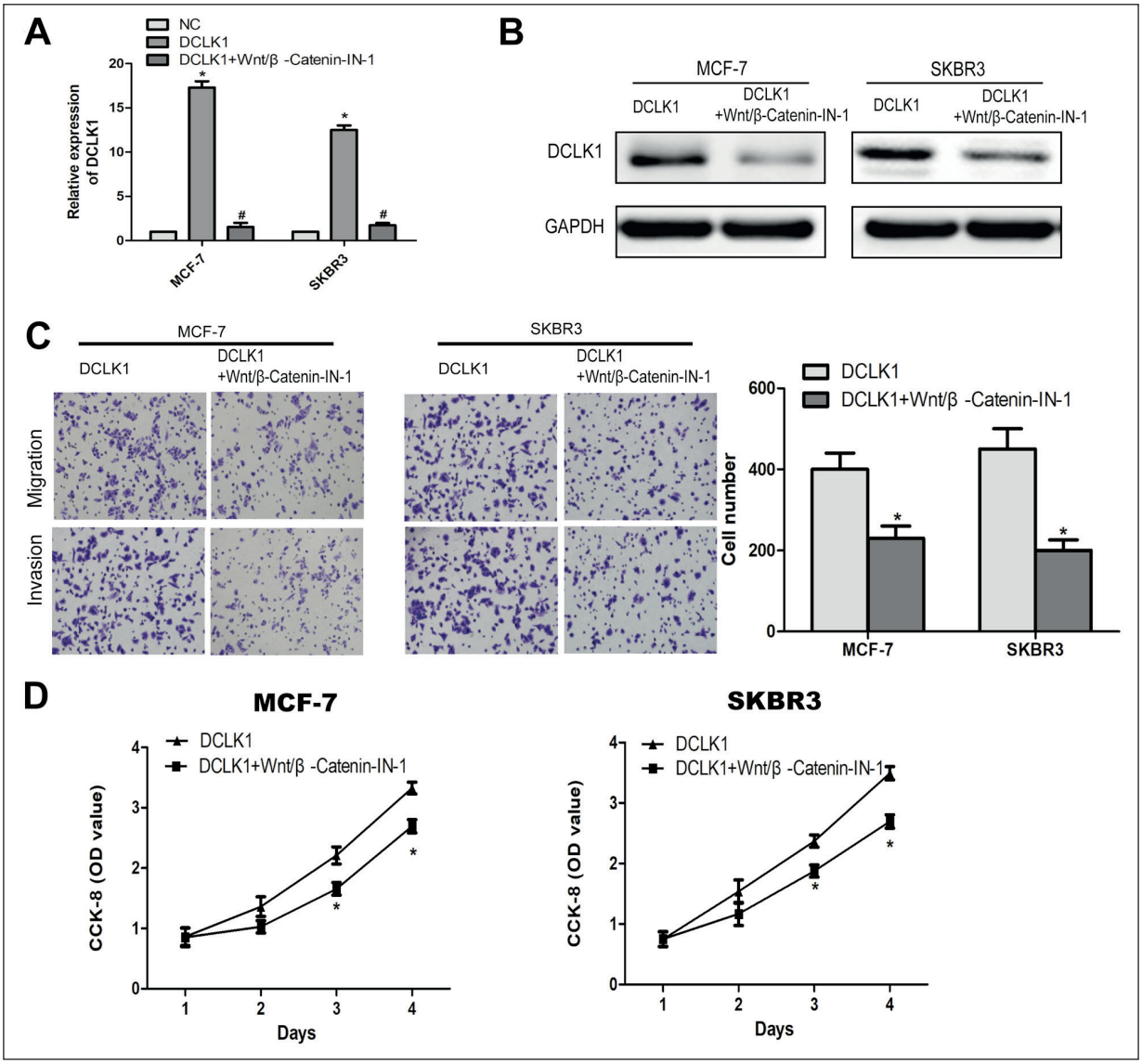

Figure 4. Wnt/β-Catenin pathway inhibitors reversed the malignant progression of breast cancer cells induced by DCLK1. **A,** qRT-PCR verified the difference in expression of DCLK1 after overexpression of DCLK1 and DCLK1+Wnt/β-Catenin-IN-1 groups in MCF-7 and SKBR3 cell lines. **B,** Western blot verified the differential expression of DCLK1 after overexpression of DCLK1 and DCLK1+Wnt/β-Catenin-IN-1 groups in MCF-7 and SKBR3 cell lines. **C,** Tranwell assay detected the effect of DCLK1+Wnt/β-Catenin-IN-1 on the invasion and migration of MCF-7 and SKBR3 cell lines (magnification: 40×). **D,** CCK-8 assay detected the effect of DCLK1+Wnt/β-Catenin-IN-1 on the proliferation of MCF-7 and SKBR3 cell lines. Data are mean ± SD, *$p<0.05$.

plays an extremely important role in the development and progression of breast cancer. To verify the impact of DCLK1 on the biological behavior of breast cancer cell lines, we performed CCK8, cell cloning, and tranwell assay on breast cancer cells with high expression or low expression of DCLK1. As a result, DCLK1 was found to be able to promote the proliferation and metastasis ability of BCa cells, but its specific molecular mechanism remains elusive.

The Wnt/β-Catenin signal pathway exists widely in cells and is a signal transduction pathway involved in the regulation of cell growth, proliferation, and differentiation. The increased activity of Wnt/β-Catenin is often associated with a variety of tumors and plays a pivotal role in the occurrence, development, treatment, and prognosis of malignant tumors[17-20]. In this experiment, to demonstrate whether DCLK1 can promote the development of BCa by regulating

the Wnt/β-Catenin signal pathway, we performed Western blot to examine the expression changes of key proteins in Wnt/β-Catenin signal pathway after the knockdown of DCLK1. The results indicated that the levels of key proteins such as β-Catenin, c-myc, and cyclinD1 in the Wnt/β-Catenin signal pathway decreased remarkably after the knockdown of DCLK1. Therefore, DCLK1 can play a critical role in promoting proliferation and metastasis of breast cancer cells through the Wnt/β-catenin signal pathway. Subsequently, we used Wnt/β-Catenin pathway inhibitor to analyze whether DCLK1 can accelerate the malignant progression of BCa through Wnt/β-Catenin pathway. The result suggested that Wnt/β-Catenin inhibitor reversed the cancer-promoting effects of DCLK1. As the research continues to deepen, further understanding of the role of genetic biology and its role in the development of tumors will be more conducive to the diagnosis and treatment of tumors.

Conclusions

We demonstrated that the DCLK1 expression was remarkably increased in BCa tissues and cells and was closely correlated with the pathological stage and poor prognosis of BCa patients. Moreover, DCLK1 may promote BCa cell proliferation, as well as inhibit its apoptosis by regulating the Wnt/β-Catenin signal pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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