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# MiRNA-106a promotes breast cancer progression by regulating DAX-1

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**Abstract. – OBJECTIVE: The aim of this study was to explore the expression of microRNA-106a in breast cancer (BC) and to further investigate its role in BC development and the potential regulatory mechanism.**

**PATIENTS AND METHODS: 72 pairs of BC tissues and para-cancerous tissues were collected, and microRNA-106a expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between microRNA-106a expression and BC pathological parameters was analyzed. Meanwhile, the expression of microRNA-106a in BC cells was verified by qRT-PCR as well. In addition, microRNA-106a knockdown model was constructed by transfecting small interfering RNA in BC cell lines including MCF-7 and SKBR3. Subsequently, the effects of microRNA-106a on biological functions of BC cells were analyzed by cell counting kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EDU), and transwell invasion and migration assays, respectively. Finally, the underlying mechanism was explored by cellular rescue experiment.**

**RESULTS: QRT-PCR results illustrated that microRNA-106a expression in BC tissues was markedly higher than that of normal tissues. Patients with high expression of microRNA-106a exhibited significantly higher tumor stage as well as higher incidence of lymph node metastasis and distant metastasis when compared with those with low expression. Cell proliferation, invasion, and migration abilities in microRNA-106a inhibitor group were markedly decreased when compared with control group. Subsequent experiments demonstrated that DAX-1 expression was reduced in BC cell lines and tissues. Moreover, DAX-1 expression was negatively correlated with microRNA-106a expression. In addition, a recovery experiment found that microRNA-106a and DAX-1 had mutual regulation, which could affect the malignant progression of BC.**

**CONCLUSIONS: We found that the expression of microRNA-106a was significantly increased in BC. Meanwhile, microRNA-106a expression was closely related to BC stage, distant metastasis, lymph node metastasis, and poor prognosis. Therefore, microRNA-106a promoted the invasion, migration, and proliferation of BC by targeting DAX-1.**

*Key Words:*
MicroRNA-106a, DAX-1, Breast cancer (BC), Proliferation, Metastasis.

## Introduction

Breast Cancer (BC) is the most common malignant tumor in women, while occasionally occurs in men. The incidence of BC in the developed countries, such as Europe and the United States, ranks first among all female malignancies. In China, the incidence of BC among female patients is also the highest, which is increasing year by year[1,2]. Therefore, BC is currently a serious threat to women's health[3]. In recent years, with the improvement of early BC diagnosis, individualized treatment has become the primary strategy for BC patients. Reasonable comprehensive application of surgery, radiotherapy, chemotherapy, biological targeted therapy, endocrine therapy, etc., has significantly improved the overall survival rate of BC patients[4-6]. Previous studies[6-8] have indicated that BC is one of the solid tumors with good prognosis. It is a complex biological process formed by multiple factors, in which genes and multiple signal pathways interact with each other. With the improvement of science and technology, such as molecular biology and genomics, the multidisciplinary crossover has triggered a rush to explore the occurrence and development of BC from molecules and genes prospectives[9].

MicroRNAs (miRNAs) are a class of endogenous, short non-coding RNAs. They are capable of regulating gene expression by complementary pairing with target mRNAs, thereby inducing their degradation[10,11]. Also, miRNA plays a significant role in cell proliferation, morphology, differentiation, and apoptosis, as well as tumor progression. MiRNAs exert their function with tissue-specific characteristics[12]. Abnormal expression of miRNA can affect the biological



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behavior of tumor cells by regulating target genes, making miRNA a new hotspot in tumor research[12-14]. MicroRNA-106a has been discovered for a long time. However, its biological function has just begun to be studied[15]. Scholars[16,17] have illustrated that microRNA-106a is expressed in multiple tumor tissues. Meanwhile, it participates in many physiological and pathological processes of tumor cells, eventually promoting tumor development. Studying the target genes of miRNAs is the core of miRNA function research. This includes how miRNAs regulate target genes, participate in regulating cell life activities, and how to influence the biological behavior of cells[9]. Therefore, the focus of miRNA research is to explore the target genes involved in the regulation of miRNAs and signaling pathways.

DAX-1 is a member of the atypical nuclear receptors family, which also plays an important role in the adrenal cortical formation and sex differentiation. DAX-1 is expressed in the human hypothalamus, pituitary gland, adrenal gland, and some other organs[18,19]. Researches[20,21] have demonstrated that DAX-1 expression in metastatic BC patients is significantly lower than that of non-metastatic patients. However, the specific mechanism of DAX-1 in BC remains unclear. Studies have shown that microRNA-106a was negatively correlated with DAX-1 expression in BC. Therefore, the aim of this study was to explore the regulation effect of microRNA-106a on DAX-1 and to investigate the mechanism by which DAX-1 was involved in the occurrence and progression of BC.

## Patients and Methods

### BC Patients and Samples

72 pairs of tumor tissues and para-cancerous tissues were collected from BC patients (aged 45-76 years) with radical mastectomy. All patients did not receive any radiotherapy or chemotherapy before surgery. Pathological classification and staging criteria for BC were performed in accordance with the International Union Against Cancer (UICC) BC staging criteria. This study was approved by the Ethics Oversight Committee of our Institution. The informed consent was obtained from each patient before the study.

### Cell Lines and Reagents

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

### Cell Transfection

Negative control (NC) and microRNA-106a inhibitor were provided by Shanghai Jima Company (Shanghai, China). Cells were seeded into 6-well plates and grown to 70% cell density. Subsequently, cell transfection was performed according to the instructions of Lipofectamine 2000 (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 Proliferation

48 h after transfection, the cells were collected and seeded into 96-well plates with 2000 cells per well. Then the cells were cultured for 6 h, 24 h, 48 h, and 72 h, respectively. Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well, followed by incubation for another 2 hours in the dark. Optical density (OD) value of each well at the wavelength of 490 nm was measured by a microplate reader. Finally, the collected data was analyzed.

### 5-Ethynyl-2'-Deoxyuridine (EDU) Dyeing

EDU proliferation assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After 24 h of transfection, the cells were incubated with 50 μm EDU for 2 h, and stained with AsoLo and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). The number of EDU-positive cells was analyzed by a fluorescence microscopy. The rate was calculated by the number of EDU positive cells to total DAPI chromogenic cells (blue cells).

### Transwell Cell Migration and Invasion

48 hours after transfection, the cells were digested with trypsin and collected in serum-free medium. After cell counting, the concentration of cells was adjusted to $2.0\times10^5$/mL. Transwell chamber with or without Matrigel was first placed in a 24-well plate. 200 μL of cell suspension was added to the upper chamber; 500 μL of medium containing 10% FBS was added to the lower

chamber. Then the cells were incubated in a 37°C incubator for 48 hours. Subsequently, the chamber was taken out and fixed with 4% paraformaldehyde for 30 minutes. After staining with crystal violet for 15 minutes, the cells were washed with phosphate-buffered saline (PBS). The inner surface of the basement membrane of the chamber was carefully cleaned to remove cells on the inner layer. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under a microscope. 5 fields were randomly selected for each sample.

### Western Blot Assay

Transfected cells were lysed using cell lysis buffer, followed by shaking on ice for 30 minutes. After centrifugation at 14,000 g for 15 minutes, the concentration of total protein was calculated using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). DAX-1 monoclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and horseradish peroxidase-labeled secondary antibodies were purchased from Genscript (Nanjing, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The protein was lysed by PRO-PREPTM protein lysate (Beijing QiWei YiCheng Tech., Ltd., Beijing, China). Extracted protein samples were separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis and transferred onto membranes. After blocking with 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with secondary antibody for 1 h (Thermo Fisher Scientific, Waltham, MA, USA). Immunoreactive bands were exposed by the enhanced chemiluminescence (ECL) method. Finally, images were semi-quantitatively analyzed using alpha SP image analysis software. This experiment was repeated for three times.

### QRT-PCR

The mRNA expressions of DAX-1, β-actin, microRNA-106a, and U6 in BC tissues and cells were detected by qRT-PCR. Total RNA was extracted by the one-step method with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed according to the instructions of Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan) reverse transcription kit. Primers were designed by Primer 5.0 software (La Jolla, CA, USA). QRT-PCR reaction was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus qRT-PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study were as follows: miRNA-106a: forward: 5'-AAAAGTGCTTACAGTGCAGGTAG-3'; U6: forward: 5'-CGCAAGGATGACACGCAAATTC-3''; DAX-1: forward: 5'-TCCGCGCCCTTGCCCAGACC-3', Reverse: 5'-GCCGCACGAACAGCCCCAACACT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Three replicates were set in each group, and this experiment was repeated twice. Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process data with software iQ5 2.0. β-actin and U6 genes were used as internal references. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis. The *t*-test was performed for measurement data, and the $\chi^2$-test or Fisher's exact probability method was used for categorical variables. Survival analysis was performed using the Kaplan-Meier method, and survival curves were plotted. Data were expressed as mean ± standard deviation ($\overline{x}\pm s$). $p<0.05$ was considered statistically significant.

## Results

### MicroRNA-106a was Highly Expressed in BC Cell Lines and Tissues

MicroRNA-106a expression in BC tissues and cell lines was first detected. QRT-PCR results indicated that the expression of microRNA-106a in BC tissues was significantly higher than that of para-cancerous tissues, and the difference was statistically significant (Figure 1A). Similarly, microRNA-106a was found remarkably higher in BC cells than normal mammary epithelial MCF-10A cells. Among 3 BC cell lines, MCF-7 and SKBR3 expressed the highest level of microRNA-106a, which were selected for subsequent experiments (Figure 1B).

### MicroRNA-106a Expression was Correlated with Clinical Stage, Lymph Node and Distance Metastasis in BC Patients

According to microRNA-106a expression, 72 pairs of BC tumor tissues and para-cancerous tissues were classified into high microRNA-106a expression group and low microRNA-106a expres-

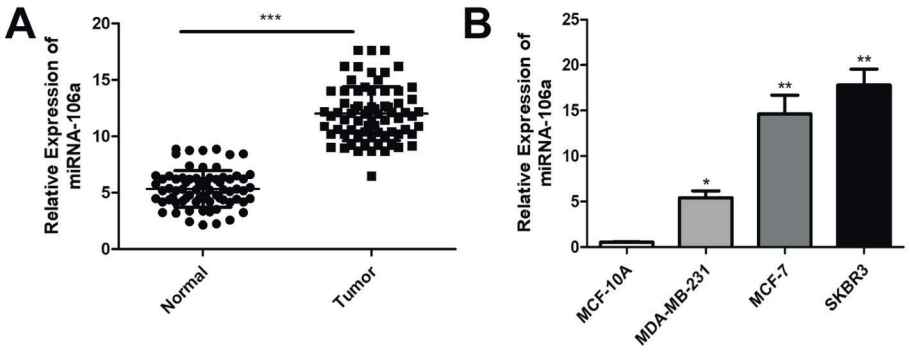


**Figure 1.** MicroRNA-106a was highly expressed in BC tissues and cell lines. ***A,*** QRT-PCR was used to detect the expression of microRNA-106a in BC tissues and para-cancerous tissues. ***B,*** QRT-PCR was used to detect the expression level of microRNA-106a in BC cell lines.

sion group. The relationship between microRNA-106a expression and age, gender, clinical stage, lymph node or distant metastasis was analyzed. As shown in Table I, high expression of microRNA-106a was positively correlated with BC clinical stage, lymph node metastasis, and distant metastasis, whereas was not correlated with age and gender. These results implied that microRNA-106a might be a biological indicator for BC prognosis.

### *Knockdown of MicroRNA-106a Inhibited BC Cell Migration, Invasion and Proliferation*

To explore the effect of microRNA-106a on BC cell function, we first constructed a microRNA-106a inhibitor model. Transfection efficiency was verified by qRT-PCR (Figure 2A). Then, we performed cell proliferation, invasion, and migration assays in SKBR3 and MCF-7 cell lines, respectively. CCK-8 and EDU assay found that the proliferation of microRNA-106a inhibitor group was significantly decreased when compared with NC group (Figures 2B and 2C). In addition, transwell assay was used to examine the effect of microRNA-106a on BC cell invasion and migration. The results revealed that, compared with the NC group, the number of BC cells transmembrane in the transwell chamber of microRNA-106a inhibitor group was significantly reduced. These findings suggested that the migration and invasive abilities of BC cells were inhibited after microRNA-106a down-regulation (Figure 2D).

**Table I.** Association of miRNA-106a expression with clinicopathologic characteristics of breast cancer.

| Parameters | Number of cases | miRNA-106a expression | | *p*-value |
|---|---|---|---|---|
| | | Low (%) | High (%) | |
| Age (years) | | | | 0.225 |
| <60 | 30 | 20 | 10 | |
| ≥60 | 42 | 22 | 20 | |
| Gender | | | | 0.217 |
| Male | 35 | 23 | 12 | |
| Female | 37 | 19 | 18 | |
| T stage | | | | 0.014 |
| T1-T2 | 41 | 29 | 12 | |
| T3-T4 | 31 | 13 | 18 | |
| Lymph node metastasis | | | | 0.049 |
| No | 43 | 28 | 13 | |
| Yes | 29 | 14 | 17 | |
| Distance metastasis | | | | 0.012 |
| No | 58 | 38 | 20 | |
| Yes | 14 | 4 | 10 | |

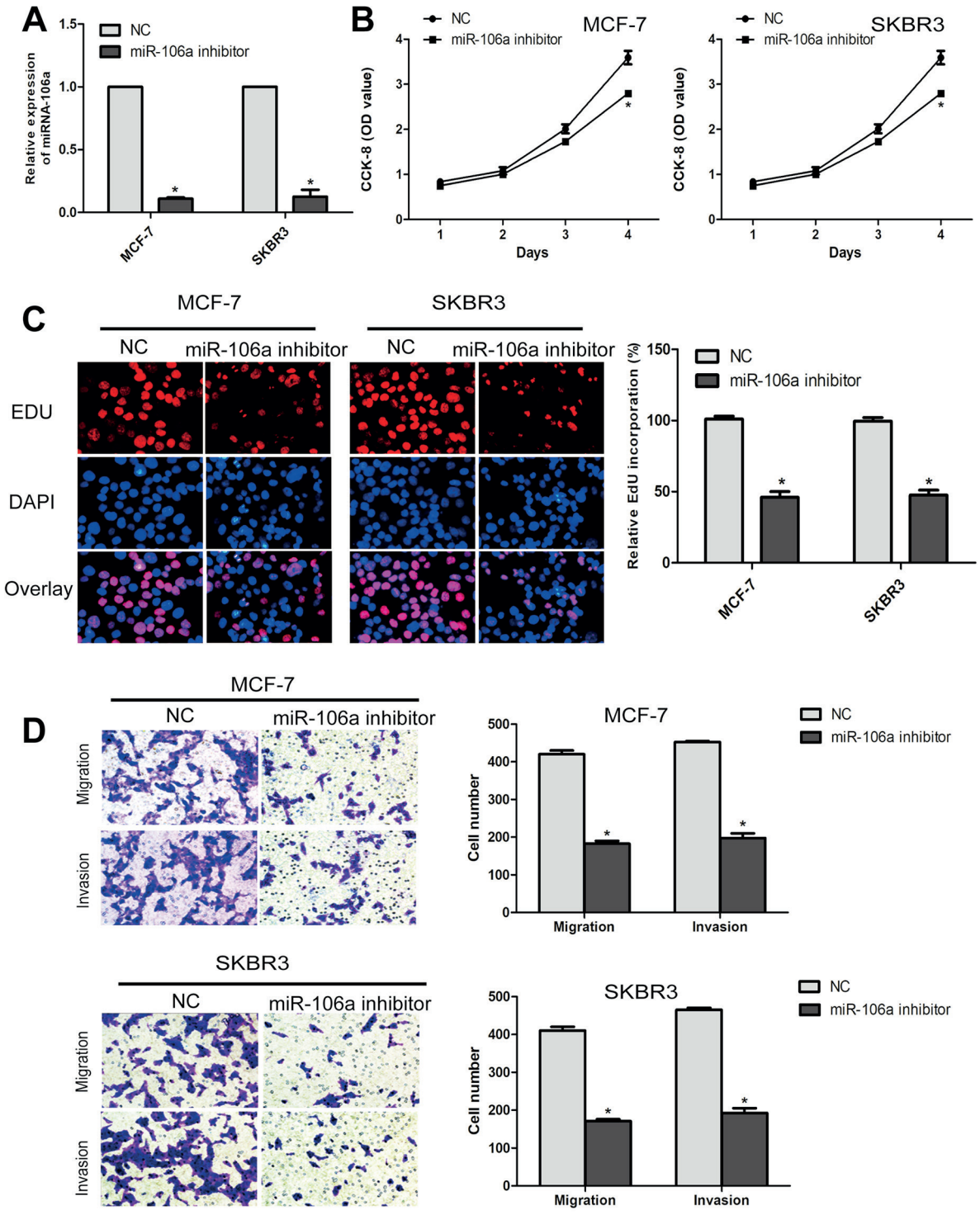


**Figure 2.** MicroRNA-106a affected cell proliferation, invasion, and migration in BC. ***A,*** QRT-PCR was used to verify the interference efficiency of microRNA-106a after microRNA-106a inhibitor transfection in MCF-7 and SKBR3 cell lines. ***B,*** CCK-8 assay was applied to explore the effects of microRNA-106a on the proliferation of MCF-7 and SKBR3 cells. ***C,*** EDU assay was used to detect the proliferation of MCF-7 and SKBR3 cells. ***D,*** Transwell migration and invasion assays were used to detect the invasion and migration of MCF-7 and SKBR3 cells. Data were expressed by mean ± SD, *$p<0.05$.

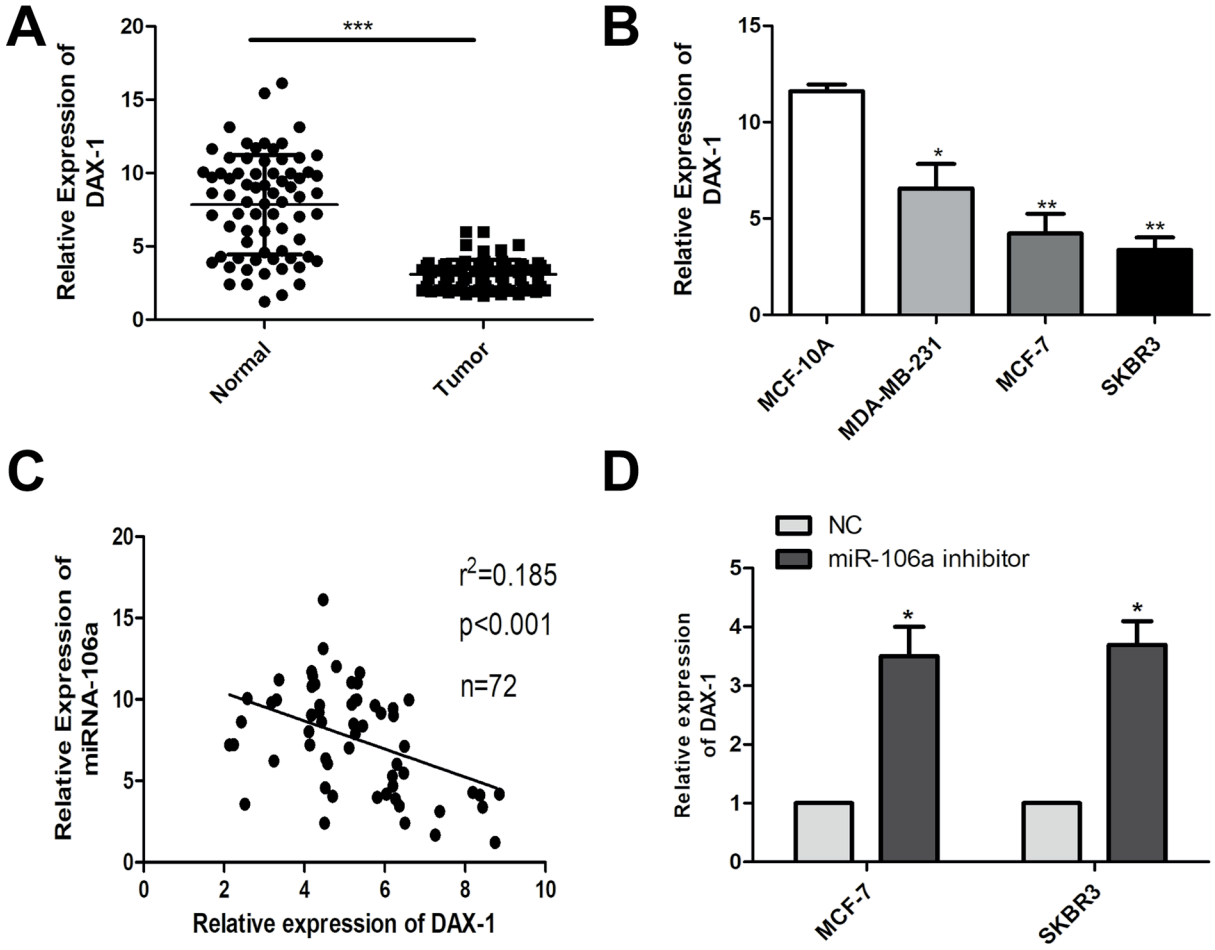


**Figure 3.** DAX-1 was low-expressed in BC tissues and cell lines. ***A,*** QRT-PCR was used to detect the expression of DAX-1 in BC tissues and adjacent non-tumor tissues. ***B,*** QRT-PCR was used to detect the expression level of DAX-1 in BC cell lines. ***C,*** A significant negative correlation was found between microRNA-106a expression and DAX-1 expression in BC tissues. ***D,*** QRT-PCR was used to verify the interference efficiency of DAX-1 after microRNA-106a inhibitor transfection in MCF-7 and SKBR3 cell lines. Data were expressed by mean ± SD, *$p$<0.05, **$p$<0.01, ***$p$<0.001.

### *DAX-1 was Lowly Expressed in BC Cell Lines and Tissues*

DAX-1 expression in BC cell lines and tissues was analyzed by qRT-PCR as well. The results illustrated that DAX-1 expression was significantly decreased in BC tissues when compared with para-cancerous tissues (Figure 3A). Compared with MCF-10A cell line, the expression of DAX-1 was significantly lower in BC cells (Figure 3B). Therefore, we detected the relationship between microRNA-106a and DAX-1 by qRT-PCR in BC tissues. The results showed that microRNA-106a was negatively correlated with DAX-1 in BC tissues (Figure 3C). Furthermore, results in cell lines were similar to those in tissues (Figure 3D).

### *DAX-1 Modulated MicroRNA-106a Expression in Human BC Cells*

To further explore how microRNA-106a promoted BC progression, we silenced DAX-1 in BC cell lines transfected with NC or microRNA-106a inhibitor to verify their function in BC. Transfection efficiency of DAX-1 was examined by qRT-PCR and Western blot (Figure 4A and 4B). Subsequently, we demonstrated that DAX-1 could counteract the function of microRNA-106a in BC cell proliferation, invasion, and metastasis through EDU assay and transwell assay (Figure 4C and 4D).

## Discussion

With the progress of the Human Genome Project, our understanding of diseases has entered the molecular level nowadays. Since microRNAs have been discovered and studied in depth, it has been demonstrated that microRNAs are widely involved in cell differentiation, proliferation,

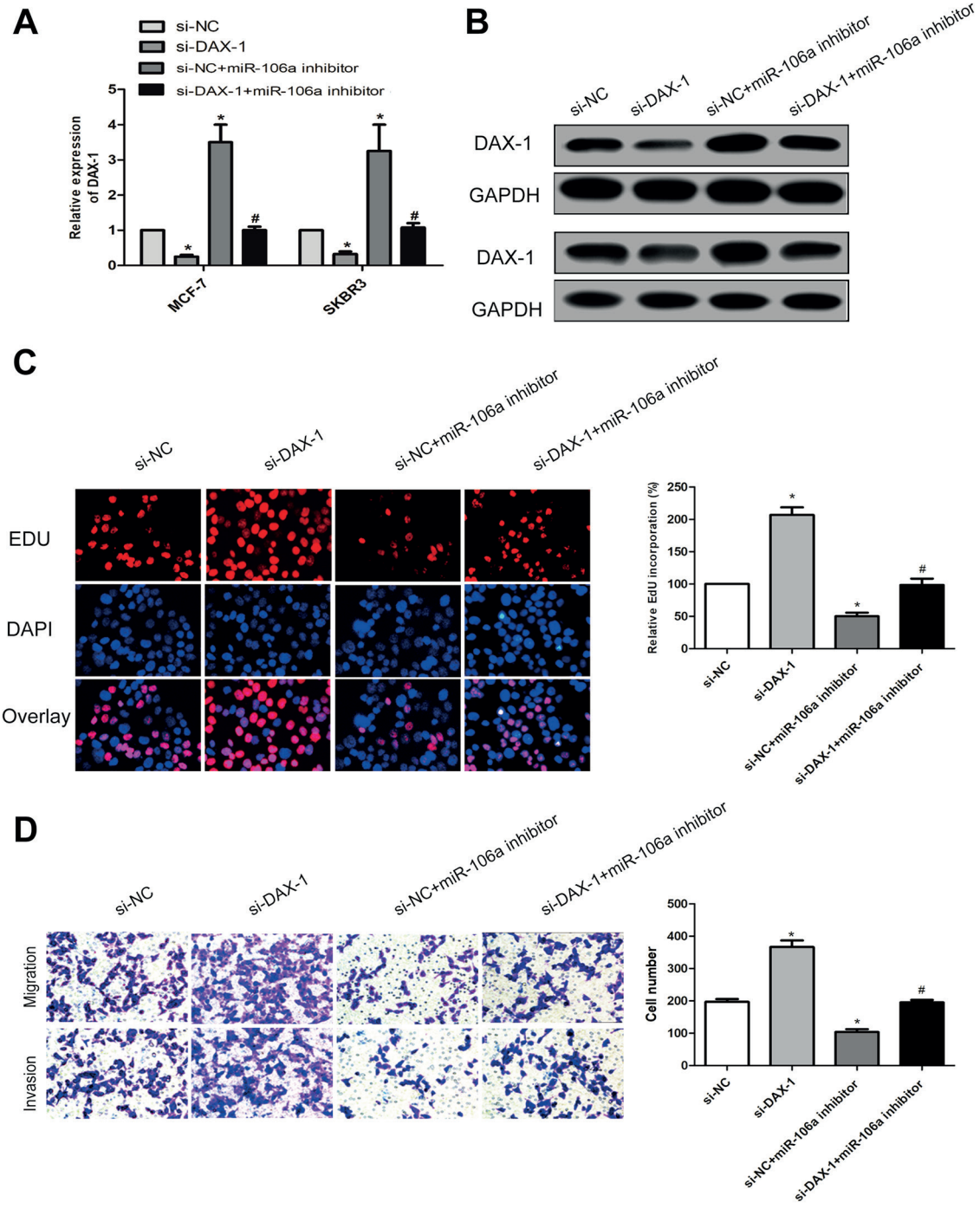


**Figure 4.** MicroRNA-106a regulated the expression of DAX-1 in BC tissues and cell lines. ***A,*** DAX-1 expression in microRNA-106a and DAX-1 co-transfected cell lines was detected by qRT-PCR. ***B,*** Western blot analysis in microRNA-106a and DAX-1 co-transfected cell lines. ***C,*** EDU assay to detect the proliferation of microRNA-106a and DAX-1 in co-transfected BC cells. ***D,*** Transwell migration assay to detect the migration and invasion of microRNA-106a and DAX-1 co-transfected BC cells. Data were expressed by average ± SD, $*^{\#}p<0.05$.

apoptosis, migration, and invasion[9,10]. Further studies have found that abnormal expression of microRNA has a certain relationship with malignant tumors. Therefore, we are paying more and more attention to exploring the role of miRNAs in tumor progression[11]. MicroRNAs are endogenous, non-coding small RNAs that can regulate gene expression by negatively targeting messenger RNAs (mRNAs)[9-11]. According to previous data, microRNAs can regulate more than 30% of human cellular processes. Increasing evidence has shown that miRNAs exert important effects on human tumor growth, invasion, and apoptosis.

A large number of studies have illustrated that abnormal expression of certain microRNAs is positively or negatively associated with malignant tumors. This suggests that microRNAs cannot be ignored in the occurrence and development of malignancies[12]. Besides, microRNAs can act as oncogenes or tumor suppressor genes in different tumors[10]. Many studies[11-13] have revealed that significant changes in microRNAs expression are associated with proliferation and metastasis of certain tumors. Although microRNA-106a has been discovered for a long time, its biological function remains elusive. It has been proved that microRNA-106a involves in the physiological and pathological processes of multiple tumors, including NSCLC, BC, and gastric cancer[22-24]. However, the exact function of microRNA-106a has not been elucidated. In this study, we demonstrated that microRNA-106a was highly expressed in BC, implying that microRNA-106a might have a potential role in BC.

To explore the function of microRNA-106a in BC development, qRT-PCR was used to determine microRNA-106a expression in 72 paired BC tissues and para-cancerous tissues. The expression of microRNA-106a was found significantly higher in BC tissues than para-cancerous tissues. Meanwhile, microRNA-106a expression was positively related to BC staging, distant or lymph node metastasis. Therefore, we speculated that microRNA-106a might contribute to the development of BC. Next, we used qRT-PCR to examine DAX-1 expression. The results illustrated that the expression of DAX-1 was remarkably down-regulated in BC tissues compared with para-cancerous tissues, and so it was the expression of microRNA-106a in BC cell lines. Among the three cell lines, the expression of microRNA-106a was found highest in MCF-7 and SKBR3 cells. Therefore, the two cell lines were selected for subsequent experiments.

MicroRNAs are RNAs with 19-24 nt in length, which can silence target gene expression post-transcriptionally by binding to target mRNAs. On a near perfect hybrid between miRNA and target complex, the target gene is cleaved and subsequently degraded. Without tight hybridization, the target can be degraded or translation is blocked[9-12]. MiRNA plays an important role in cell proliferation, morphology, apoptosis, and differentiation. In addition to studies on miRNA silencing mechanisms, the regulation of miRNA is also an important challenge[9,10]. Therefore, it is extremely important to study the potential target genes of microRNAs. It has been found that the human genome contains more than 1000 microRNA target genes. Nearly one-third of protein-coding genes are regulated by microRNAs[11-13]. To further explore the effect of microRNA-106a on the biological function of BC, we constructed a microRNA-106a knockdown expression model using lentivirus. CCK8, EDU, transwell invasion, and migration experiments demonstrated that microRNA-106a could significantly promote the development of BC. However, its specific molecular mechanism is still unclear.

The study of miRNA target genes remains the core of miRNA function research. Sun et al[9] have indicated that microRNAs are involved in life activities through regulating target genes. Therefore, the focus of miRNA research is to discover and clarify target genes regulated by microRNAs and involved signaling pathways[10]. In recent years, researches on microRNAs have greatly deepened. Conceptual and technological innovations lead to an expanding understanding of biology and molecular pathology of tumors[25,26]. MiRNA binds to the 3'-UTR region of target genes through base-pair pairing, which degrades target protein or reverses translation[26,27]. A certain miRNA can regulate multiple target genes, while different target genes can also participate in the same signaling pathway[27]. To clarify the biological function of miRNAs, we need to further search for their target genes. Moreover, it is of great significance to explore its role and influence on the tumor development process. In the present study, results indicated that microRNA-106a inhibitor transfection significantly up-regulated the mRNA and protein expression of DAX-1. Subsequently, a recovery experiment was conducted to verify that DAX-1 counteracted the role of microRNA-106a in the malignant progression of BC.

## Conclusions

We observed that microRNA-106a was highly expressed in BC. Meanwhile, its expression was significantly correlated with BC stage, distant or lymph node metastasis, and poor prognosis of patients. In addition, microRNA-106a could promote the development of BC by acting on DAX-1.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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