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Downregulation of microRNA-645 suppresses breast cancer cell metastasis via targeting DCDC2

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Abstract. – OBJECTIVE: To analyze the functioning mode of miR-645 on breast cancer cell metastasis and provide therapeutic targets for breast cancer.

MATERIALS AND METHODS: Quantitative Real-time PCR (qRT-PCR) assay was employed to detect miR-645 expression level. Wound healing assay and transwell assay were performed to investigate metastasis capacity of breast cancer cells. Protein levels were assessed by Western blotting assay. The target gene was predicted and verified by bioinformatics analysis and luciferase assay.

RESULTS: MiR-645 was upregulated in breast cancer tissues when compared with pericarcinous tissues (n=60). Downregulated miR-645 could attenuate breast cancer cell migration and invasion capacities, as well as inhibit the process of epithelial-mesenchymal transition (EMT). DCDC2 was chosen as the target gene of miR-645 by bioinformatic analysis and Luciferase reporter assay. Moreover, the silence of DCDC2 could rescue tumor suppression role of downregulated miR-645 on breast cancer metastasis.

CONCLUSIONS: Knockdown of miR-645 exerted tumor-suppressive effects on breast cancer metastasis via targeting DCDC2 *in vitro,* which provided an innovative and candidate target for diagnose and treatment of breast cancer.

Key Words:
microRNAs, Metastasis, EMT, DCDC2, Breast cancer.

Introduction

Breast cancer (BC) is one of the most common malignancies in women[1]. Epidemiological survey data show that the incidence rate ranks second in the United States, and the mortality rate has ranked first among cancer-related mortality rate[2]. In China, the incidence rate of BC is low, but shows an increasing trend year by year in some large and medium cities, such as Beijing and Shanghai[3]. The pathogenesis of BC is still unclear so far, and it is thought that the occurrence and development of BC are associated with a variety of factors, such as genetic factors, endocrine factors and malignant benign breast lesions[4]. Although there are various treatment methods at present and the initial curative effects of traditional surgery, chemotherapy and other treatment measures are efficient, the treatment often fails due to the malignant biological behaviors of BC, such as easy relapse and early metastasis[5,6], so it is particularly important to deeply focus on the formation mechanism of malignant biological behaviors of BC.As a type of small non-coding ribose nucleic acid (RNA) transcripts, microRNA is endogenous RNA with 18 to 25 nucleotides approximately in length[7]. It suppresses gene expression through post-transcription in various biological processes[8]. It can bind to the 3'UTR of their target genes to suppress protein translation[9]. More and more studies[10] discovered that microRNA could play an important role in various cell progressions. Therefore, well study of microRNA may be of great value in explaining the occurrence and development of tumors. MicroRNA-645, a tumor-related miRNA, has been documented to be downregulated and plays an anti-oncogene role in several kinds of cancers, such as colon cancer[11], rectal cancer[12], head and neck cancer[13] and gastric carcinoma[14]. Meanwhile, it has reported that miRNAs participated in the process of BC tumorigenesis[15]. However, the mechanism of action about miR-645 in development and progression of BC remains unknown. In the present work, we identified that miR-645 expression was increased in BC tissues. Meanwhile, downregulated miR-645 could suppress BC cell migration and invasion capacities *in vitro*, and inhibit epithelial-mesenchymal transition

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(EMT) process. Further studies demonstrated that miR-645 could exert functions by modulating its target gene DCDC2.

Material and Methods

Clinical Samples

60 pairs of BC tissues and matched pericarcinous tissues were obtained from patients undergoing routine surgery in Jilin Cancer Hospital from 2015-2017. All surgical specimens were collected and then frozen immediately in liquid nitrogen until use. Tumor tissues were diagnosed and confirmed by pathological examination. This research was approved by the Ethics Committee of Jilin Cancer Hospital. Written informed consents were signed from all participants before the study.

Cell Culture

The BC cell lines LCC2, LCC9, MCF-7, T-47D, SKBR3, MDA-MB-453, DU4475 and normal human breast cell line, MCF-10A, were purchased from Shanghai Model Cell Bank (Shanghai, China). Cells were cultured in media (Roswell Park Memorial Institute-1640) RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 ug/mL streptomycin. All cells were cultured in an incubator at 37°C with 5% CO_2.

Plasmid and Transfection

For downregulation of miR-645 in BC cells, miR-645 inhibitor and corresponding negative control (inhibitor-NC) were obtained from the RiboBio (Guangzhou, China). Transfections were performed using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. For knockdown of DCDC2 for silencing, siRNA sequences that targeting for DCDC2 were designed as follows: siRNA-1: 5'-CAG TGG ACA AGA TGA GGC TGA TGT A-3'; siRNA-2: 5'- CCA GAA AGT CTA AAG GGA GTG GAA A-3'; siRNA-3: 5'-GGG CTG TTC ACA GGC TTT ATA CTT T-3' and the siRNA-NC were purchased from Genechem (Shanghai, China).

RNA Extraction and qRT-PCR

Total RNA was extracted from collected frozen BC tissues and matched paracarcinoma tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. The relative expression level of miR-645 was determined using the mirVana™ qRT-PCR microRNA Detection kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols. U6 was used for normalization. Then, we performed PCR reactions using the following primers: for miR-645, forward, 5'-AGA CAG TGG CAA TAC TGC UCA-3' and reverse, 5'-GGT CCG GTG CAG AGG T -3'; and for U6, forward, 5'-GCA CCT TAG GCT GAA CA-3' and reverse, 5'-AGC TTA TGC CGA GCT CTT GT-3'. PrimeScript® RT reagent kit (TaKaRa, Dalian, China) was used to synthesize cDNAs. The relative mRNA expression level of DCDC2 was measured by SYBR Green Real-time PCR and normalized to GAPDH using the following primers: for DCDC2, forwards, 5'-ACC TTG ATT CTG CCC GTA GC-3' and reverse, 5'- AAG TTT TTC ACC GTG GCG TG-3'; and for GAPDH, forward, 5'-CGG AGT TGT TCG TAT TCG G-3' and reverse, 5'-TAC ATG ATG TGG ACG GCA TT-3'. QRT-PCR was carried out by utilizing the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Wound Healing Assay

Transfected cells were cultured in the 6-well plates marked by a horizontal line on the back. Cells were scratched by a pipette tip across the confluent cell layer, they were washed gently and continued to culture with the serum-free medium for 24-48 h. Wound closure was captured using a light microscope (DFC500, München, Germany).

Transwell Assay

Cells were cultured in the upper invasion chamber (BD, Franklin Lakes, NJ, USA) coated with Matrigel. Serum-free medium was added into the upper chamber, whereas 10% FBS medium supplemented was added into the lower. After 48 h, cells were cultured on the upside of the filter, which did not invade through the chamber and was removed. Next, the chamber was suspended by 100% precooling methanol, stained with 0.05% crystal violet and inspected with the microscope (Olympus, Tokyo, Japan). The values for the invasion cells were measured by counting five fields per membrane.

Bioinformatics Analysis

TargetScan (http://www.targetscan.org/vert_71/) and starBase v2.0 (http://starbase.sysu.edu.cn/index.php) were utilized to forecast the target genes. As shown in the database, DCDC2

was the candidate gene we chose. The result of bioinformatics software indicated that 3'-UTR of DCDC2 binds to miR-645. Then qRT-PCR was performed to detect whether DCDC2 was really inversely correlated with miR-645 expression in BC cells. Kaplan Meier-plotter (http://kmplot.com/analysis/index.php?p=service) database were also utilized to forecast the prognosis of BC patients with ectopic expression of DCDC2.

Luciferase Reporter Assay

The activity of luciferase was tested using the Dual-Luciferase reporter system (Promega, Madison, WI, USA). The DCDC2 3'-UTR region containing the wild type or mutant miR-645 binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Treated cells were co-transfected with the established vector and miR-645 mimics or scrambled using lipofectamine 3000. Then, the activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Western Blot Analysis

To investigate relative protein expression level, cells were lysed and the concentration of collected protein was measured using a protein assay kit purchased from Beyotime (Haimen, China). The extracted protein (sum of 20 μg) was degenerated and chilled on ice. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein, and it was shifted to polyvinylidene fluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in Tris Buffered Saline Tween (TBST) buffer, which contains Tris-HCl (50 mM), NaCl (150 mM) and Tween 20 (0.05%) at 4°C for 1 h. The membranes loaded with proteins were incubated at 4°C within the fat-free milk overnight with the following primary antibody against DCDC2, E-cadherin, N-cadherin and Vimentin (Absci, Nanjing, China). TBST buffer was used to wash the unbound antibody (10 min each time for three times). Then, secondary antibody was employed to incubate the membranes conjugated with horseradish peroxide (HRP) (1 h) at room temperature. After washing these membranes three times in tris buffered saline-tween (TBST), we developed the membranes using enhancedchemiluminescence (ECL) (Millipore, Billerica, MA, USA) following the instructions.

Statistical Analysis

Statistic Package for Social Science (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Statistical data was presented with GraphPAD prism software (La Jolla, CA, USA), and quantitative data was presented as mean ± SD. The regression and correlation analysis were analyzed using the Spearman x^2 test. The relative expression of mRNA was measured using the method of $2^{-\Delta\Delta CT}$. $p < 0.05$ was seemed as statistically significant.

Results

MiR-645 Expression Was Elevated in BC Tissues and Cell Lines

Expression of miR-645 was detected in 60 pairs of BC tissues and pericarcinous tissues by qRT-PCR. The result indicated that miR-645 expression was remarkably increased in BC tissues compared with the paired pericarcinous tissues on mRNA level (Figure 1A). This evidence implied that miR-645 might participate in BC tumorigenesis. Then we investigated expression of miR-645 in several BC cell lines and normal breast cell line with qRT-RCR. It showed that, compared with MCF-10A cell line, all these BC cell lines expressed a relatively higher level of miR-645, in which MCF-7 expressed the relatively highest (Figure 1B). To identify the mode of action of miR-645 in BC tumorigenesis *in vitro*, MCF-7 cell line was transfected with miR-645 inhibitor and inhibitor-NC for knockdown of miR-645(Figure 1C).

Knockdown of miR-645 Inhibited BC Cell Metastasis In Vitro

We next evaluated the action role of miR-645 in BC cell metastasis *in vitro*. As shown in wound-healing assay, downregulated miR-645 could suppress BC cell migration when compared with inhibitor-NC (Figure 2A). Meanwhile, influence of downregulated miR-645 on cell invasion measured by using transwell assay was the same as the former (Figure 2B). The results demonstrated that downregulated miR-645 could inhibit cell metastasis of BC.

Knockdown of miR-645 Inhibited EMT Process

In order to further reveal the role of miR-645 in BC metastasis, Western blot was assessed to detect the expression levels of EMT-related

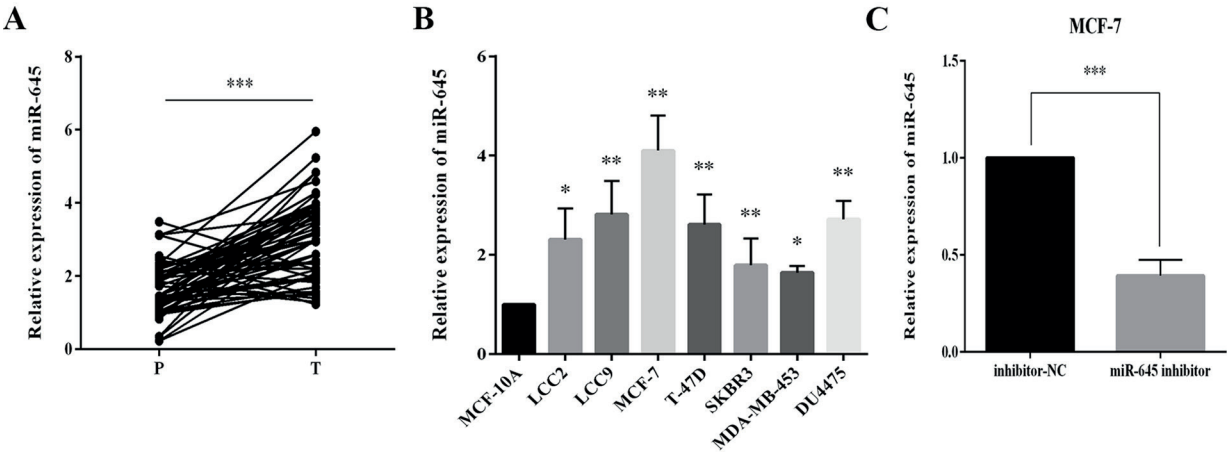

Figure 1. MiR-645 expression was decreased in BC tissues and cell lines. ***A:*** Analysis of miR-645 expression in paracarcinoma tissues (P) and tumor tissues (T); ***B:*** Analysis of miR-645 expression in several BC cell lines and normal cell line; ***C:*** Analysis of transfection efficiency in MCF-7 cells transfected with miR-645 inhibitor and inhibitor-NC. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

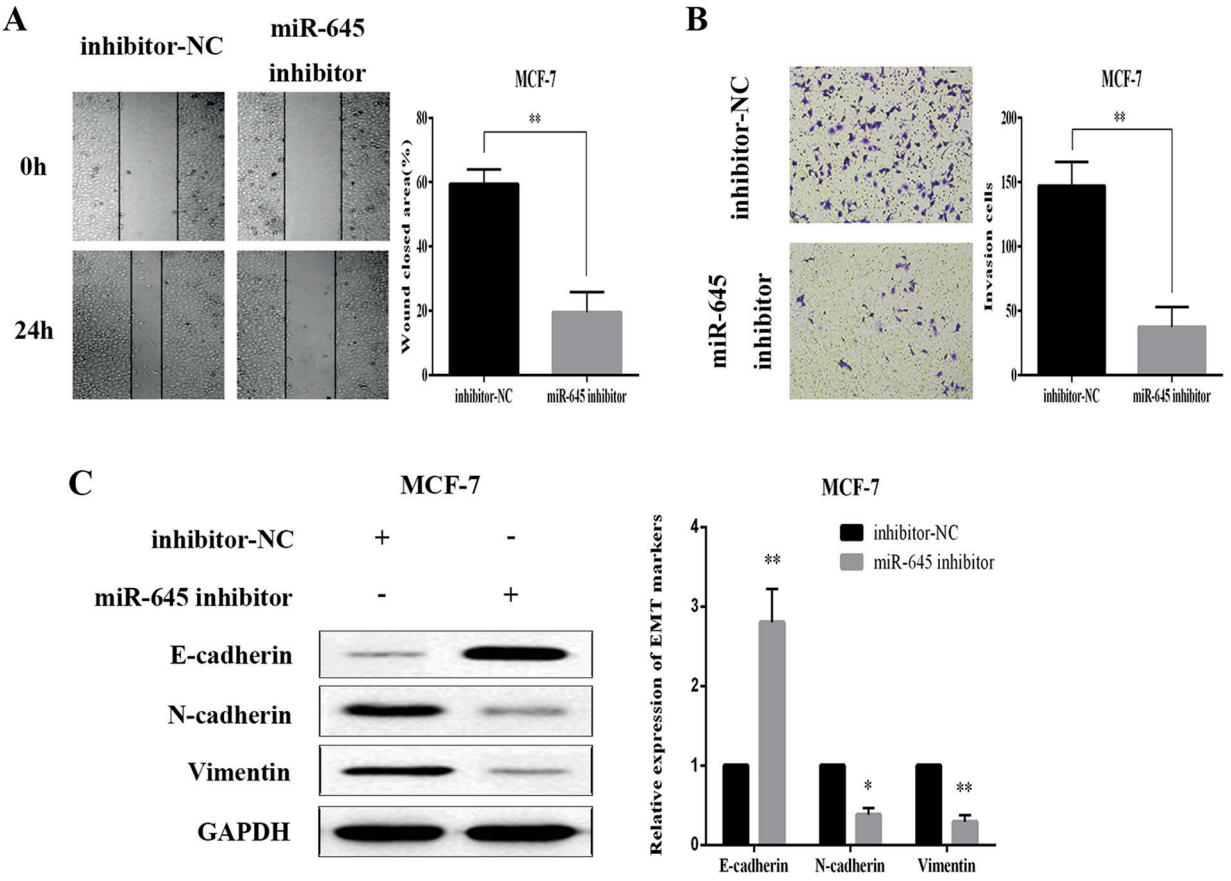

Figure 2. Knockdown of miR-645 inhibited BC cell metastasis *in vitro*. ***A:*** Wound-healing assay was performed to determine the migration of transfected MCF-7 cells; ***B:*** Transwell assay was performed to determine the invasion of transfected MCF-7 cells. ***C:*** Western blot assay was assessed to identify the expression levels of EMT-related molecular markers. *$p < 0.05$; **$p < 0.01$.

molecular markers in BC cell lines. The results showed that the expression of E-cadherin was increased in miR-645 downregulated MCF-7 cells, while the expression of N-cadherin and Vimentin were markedly decreased (Figure 2C). These results suggest that downregulated miR-645 could inhibit the process of EMT and might have a close relationship with the metastatic ability of BC.

DCDC2 is Directly Targeted by miR-645

To better understand the mechanism about how miR-645 participated in these biological processes, we selected DCDC2 as the potential downstream of miR-645 via using TargetScan and starBase database (Figure 3A). According to the consequence of prediction, miR-645 was transfected with DCDC2 3'UTR luciferase reporter gene into MCF-7 cell. The result of dual-luciferase assay displayed a significant activity decrease in the WT group but no difference in mutant group (Figure 3B), indicating that DCDC2 was a target of miR-645. Meanwhile, we further detected expression level of DCDC2 in transfected BC cells. The results indicated that DCDC2 was upregulated in MCF-7 cells transfected with miR-645 inhibitor on mRNA level and protein level

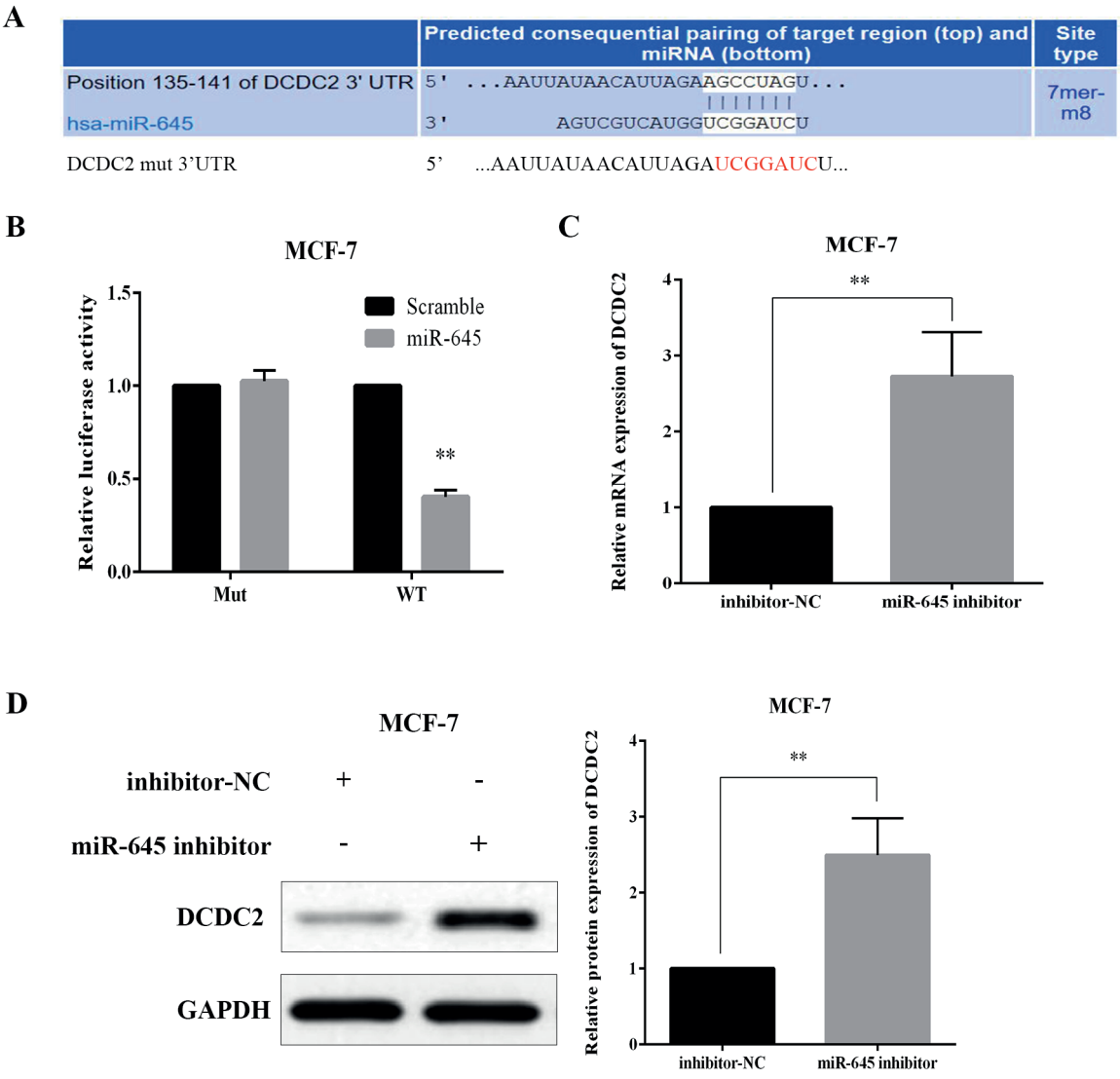

Figure 3. DCDC2 is the target gene of miR-645. ***A:*** DCDC2 was selected as the potential downstream of miR-645 via using bioinformatics analysis; ***B:*** Luciferase activities of MCF-7 cells transfected with the wild-type or the mutated DCDC2 3′UTR; ***C:*** Analysis of DCDC2 mRNA expression level of MCF-7 cells transfected with miR-645 inhibitor or inhibitor-NC; ***D:*** Analysis of DCDC2 protein expression level of MCF-7 cells transfected with miR-645 inhibitor or inhibitor-NC. Data are presented as the mean ± SD of three independent experiments.**$p < 0.01$.

when compared with inhibitor-NC (Figure 3C-D). All these results indicated that DCDC2 was directly targeted by miR-645.

Silencing of DCDC2 Rescued Tumor Suppression of Downregulated miR-645

To future identify the interaction relationship of miR-645 and DCDC2, we firstly measured the expression of DCDC2 in BC tissues. The results indicated that DCDC2 was downregulated in BC tissues compared with the paraneoplastic tissues on the mRNA level (Figure 4A), and the expression of DCDC2 was negatively correlated with the expression of miR-645 in BC tissues (Figure 4B). Moreover, we also identified that overexpression of DCDC2 led to a potential trend to prolong the survival rate of BC patients using Kaplan Meier-plotter database analysis (Figure 4C). Secondly, we explored whether DCDC2 is responsible for the functional effects of miR-645 in BC tumorigenesis. We silenced DCDC2 expression by transfected with siRNA-DCDC2

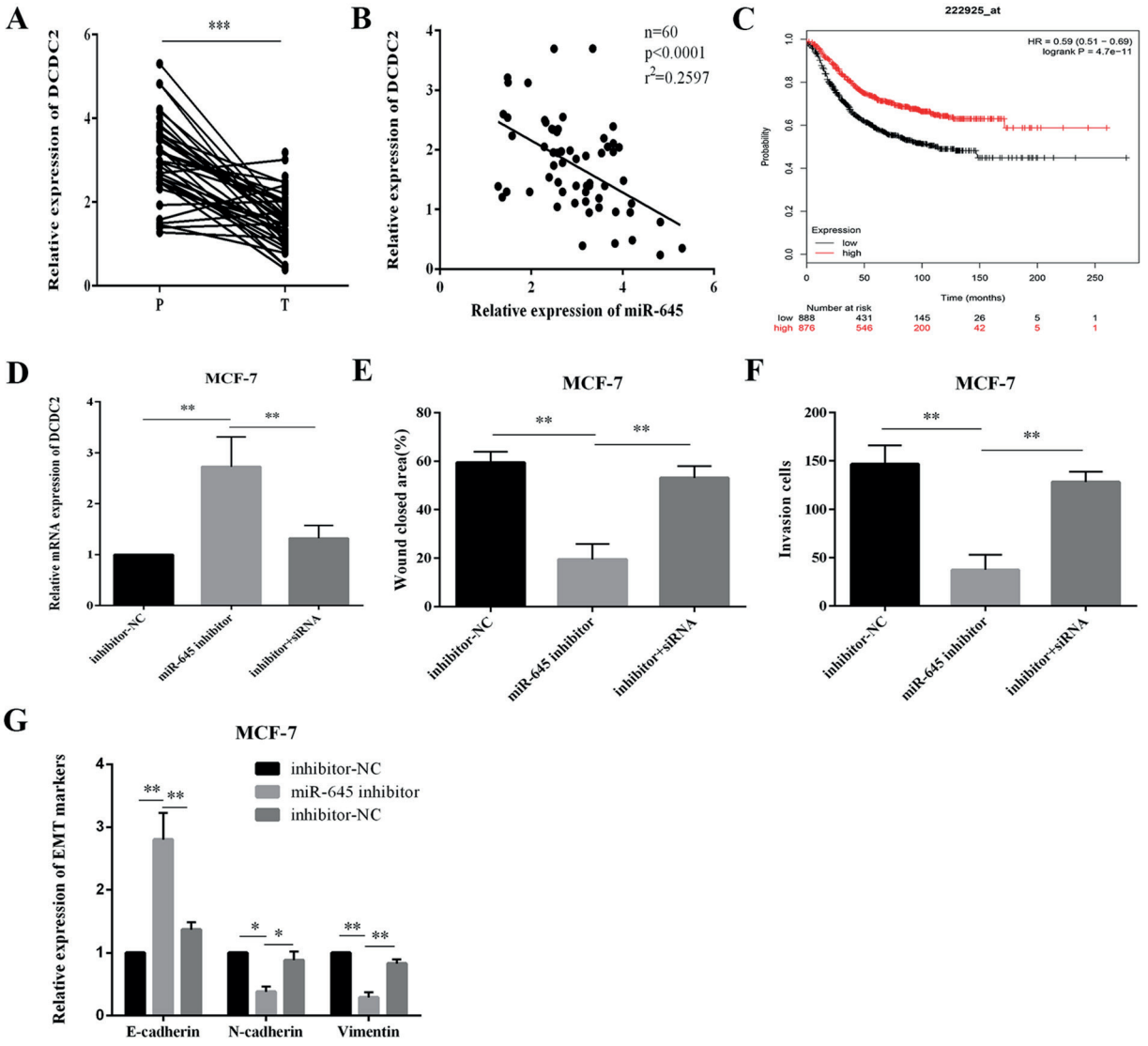

Figure 4. Silencing of DCDC2 rescued tumor suppression of downregulated miR-645. ***A:*** Analysis of DCDC2 expression level in BC tissues(T) and matched paracarcinoma tissues (N), n=60; ***B:*** Correlation between miR-645 and DCDC2 expression in BC tissues(n = 60); ***C:*** Relationship between DCDC2 and prognosis of patients with BC; D: Analysis of transfection efficiency in MCF-7 cells transfected with miR-645 inhibitor-NC, inhibitor and/or siRNA-DCDC2; ***E:*** Downregulated DCDC2 increased cell migration of miR-645-transfected cells; F: Downregulated DCDC2 increased cell invasion of miR-645-transfected cells; ***G:*** Downregulated DCDC2 promoted EMT process of miR-645-transfected cells. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

in miR-645-decreased MCF-7 cells (Figure 4D). DCDC2 silencing not only increased cell migration and invasion compared with miR-645 inhibitor (Figure 4E-F), but also promoted the process of EMT (Figure 4G). These results implied that miR-645 promoted BC tumorigenesis by repressing DCDC2 expression partially.

Discussion

Increasing studies suggest that microRNAs play a crucial role in carcinogenesis and cancer progression of various types of tumors. For example, Xia et al[16] revealed that miR-22 could suppress the development and differentiation of colorectal cancer cells and miR-22/Sp1/PTEN/AKT axis might represent a potential therapeutic target for colorectal cancer. Xiang et al[17] found that miR-93-5p could inhibit apoptosis, invasion, and switch EMT to MET phenotype through regulation of MKL-1 and STAT3 in breast cancer. Xiao et al[18] demonstrated that miR-100 might suppress osteosarcoma cell growth and decrease osteosarcoma cell chemo-resistance by targeting ZNRF2. Du et al[19] reported that miR-543 promoted prostate cancer cell growth and metastasis via targeting RKIP.BC has always been the serious threat to women in the world. In recent years, increasing studies implied that microRNAs played an important role in the genesis and development of BC. For example, Wang et al[20] demonstrated that miR-766 could function as a novel tumor suppressor by enhancing p53 signal pathway. Li et al[21] revealed that miR-148a functioned as a pivotal regulator in BC through targeting BCL-2. Jones et al[22] found that miR-200c could serve as a regulator of mesenchymal tumor cell growth via regulating expression of FLT1 and VEGFC. To date, there has not been any study of the relationship between miR-645 and BC tumorigenesis. In our investigation, we demonstrated that miR-645 was upregulated in BC tissues when compared with pericarcinomatous tissues, which implying that miR-645 might play a potential and vital role in the development of BC. Besides, downregulated miR-645 attenuated BC cell invasion and migration capacities, as well as inhibited EMT process. All these findings suggested that downregulated miR-645 exerted its suppressive effect on cell metastasis of BC. To further identify the underlying mechanism of how downregulated miR-645 inhibited BC cell tumorigenesis and metastasis, we predicted and selected DCDC2 as the novel target gene of miR-645 by bioinformatic analysis. Doublecortin domain-containing protein 2 (DCDC2), which in humans is encoded by the DCDC2 gene, is located at 6p22.3[23]. The protein encoded by the gene contains two microtubule-associated protein peptide domains, which have been proved to bind tubulin and enhance microtubule polymerization[24]. The mutation of gene was originally found to be related to reading disorder (RD), also known as developmental dyslexia[25]. Recent reports have found and confirmed that DCDC2 is closely related to the development of tumors, which can inhibit the progression of liver cancer[26], urothelial tumor[27] and prostate cancer[28]. However, the underlying upstream mechanism of DCDC2 in BC has not been well identified and reported yet. In our present study, we initially revealed that DCDC2 was directly targeted by miR-645, and DCDC2 expression was negatively correlated with miR-645 in BC tissues and cell lines. Meanwhile, overexpression of DCDC2 led to a potential trend to prolong the survival rate of BC patients. Moreover, silencing of DCDC2 could rescue tumor suppression role by downregulated miR-645 on BC cell metastasis. The evidence indicated that miR-645 might be the upstream of DCDC2 involved in BC tumorigenesis and metastasis.

Conclusions

We demonstrated that downregulated miR-645 had tumor-suppressive effect on BC metastasis via targeting DCDC2 *in vitro*. Our findings may help elucidating the molecular mechanisms underlying BC progression and provide miR-645 as an innovative and candidate target for diagnose and treatment of BC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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