

Original Article

DLC-3 suppresses cellular proliferation, migration, and invasion in triple-negative breast cancer by the Wnt/ β -catenin pathway

Bin Kong¹, Zhi-Dong Lv¹, Jing Xia¹, Li-Ying Jin², Zhao-Chuan Yang³

¹Breast Center, ²Cerebrovascular Disease Research Institute, ³Department of Child Health Care, The Affiliated Hospital of Qingdao University, Qingdao 266003, P. R. China

Received November 18, 2018; Accepted February 22, 2019; Epub April 1, 2019; Published April 15, 2019

Abstract: Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype. Our study investigated the functional role of DLC-3 in TNBC. The expression of DLC-3 was assessed by immunohistochemistry in TNBC to evaluate the clinicopathologic significance of DLC-3. Recombinant lentiviral vectors encoding the DLC-3 gene were constructed for transfection into MDA-MB-231. Real-time qPCR and western blot analysis were employed to evaluate the expression of DLC-3, β -catenin, GSK-3 β and c-myc in DLC-3-transfected cells. Moreover, cell proliferation assays, cell colony formation assays, and cell migration and invasion assays were performed to elucidate the role of DLC-3 in TNBC development and progression. Our data revealed that DLC-3 was downregulated in TNBC, and its expression level was associated with lymph node status and differentiation grade in breast cancer. Both real-time qPCR and western blot analyses showed that the DLC-3 gene and protein were overexpressed in the DLC-3-transfected MDA-MB-231 cells. In addition, the expression of GSK-3 β was upregulated and the expression of β -catenin and c-myc gene was downregulated in the DLC-3-transfected cells. Furthermore, DLC-3 overexpression inhibited cell proliferation, colony formation, migration, and invasion in vitro. DLC-3, functioning as a tumor-suppressor gene, inhibits cell growth and invasion in TNBC, possibly through regulation of the Wnt/ β -catenin signaling pathway.

Keywords: Triple-negative breast cancer, DLC-3, proliferation, invasion

Introduction

Triple-negative breast cancer (TNBC) is an invasive type of breast carcinoma that lacks expression of the estrogen receptor and progesterone receptor as well as human epidermal growth factor receptor-2 amplification [1]. We have previously reported that patients with TNBC have a relatively poorer outcome for rapid proliferation, early metastasis, and lack of molecular targets for treatment [2].

Rho-GTPases are important molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells. They play a pivotal role in regulating the actin cytoskeleton and also influence cell polarity, microtubule dynamics, membrane transport pathways, and transcription factor activities [3]. The activity of Rho-GTPases varies between the active GTP-bound state and the inactive GDP-bound state and regulates a number of cellular processes such as cytoskeleton reorganiza-

tion, gene transcription, and cell cycle progression [4]. Upregulation of the expression and activity of Rho-GTPases have been confirmed to be associated with a great number of human carcinomas [4]. Rho-GTPase-activating proteins (Rho-GAPs) have three subgroups: deleted in liver cancer gene 1-3 (DLC1-3). DLC-1 and DLC-2 genes have been shown to be downregulated in cancer cells and inhibit cancer cell proliferation [5]. DLC-3, also known as STARD8 and KIAA0189, is another Rho-GAP that maps to chromosome Xq13, which was first isolated from a human myeloid cell line library in 1996 [6]. DLC-3 consists of a sterile alpha motif (SAM), GAP (GTPase-activating protein) and START (Steroidogenic acute regulatory protein [StAR]-related lipid transfer) domain which has been reported to be downregulated in some carcinomas, such as ovarian, liver and prostate cancer cell lines [6-9]. However, the role of DLC-3 in TNBC is still unclear. In the present study, we aimed to investigate the role of DLC-3 in tumor growth and invasion and explore its

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possible interactions with the Wnt signaling pathway.

Materials and methods

Clinical samples

100 pairs of TNBC and adjacent non-cancerous tissue samples were obtained from patients who underwent modified radical mastectomy in the Affiliated Hospital of Qingdao University. The matched non-cancerous adjacent tissues were harvested at least 5 cm away from the tumor site. None of patients had received chemotherapy, radiotherapy and other related anti-tumor therapies before surgery. Written informed consent was obtained from all participants, and research protocols for the use of human tissue were approved by and conducted in accordance with the policies of the Institutional Review Boards at Qingdao University. The histologic subtype was determined according to the World Health Organization classification. The Tumor Node Metastasis (TNM) stage was determined post-operatively according to the American Joint Committee on Cancer (7th edition), and the histologic grade was determined according to the Scarff-Bloom-Richardson grading system.

Cell cultures and transfection

Three breast cancer cell lines with a basal-like transcriptional profile (MDA-MB-468, MDA-MB-231 and HCC-1937) and a human breast epithelial cell line (MCF-10A) were obtained from the Cancer Research Institute of Beijing, China. These cells were cultivated in T75 tissue culture flasks in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in humidified incubator containing 5% CO₂ at 37°C. The pcDNA3.1(+)-GFP plasmid, oligo DNA, Pfu DNA polymerase, packaging plasmids (pHelper1.0/pHelper2.0), lentiviral vectors and other routine molecular reagents were purchased from Gemma Co., Ltd. (Shanghai, China).

Construction of the expression plasmid and lentiviral vector

A full-length DLC-3 cDNA was subcloned into the pcDNA3.1(+)-GFP plasmid by using T4 DNA ligase, constructing the recombinant plasmid pcDNA3.1(+)-GFP-DLC-3. The sequence and orientation of the vector inserts were confirm-

ed by restriction enzyme digestion and DNA sequencing. Next, competent cell line Top10 was transfected with the recombinant plasmid using Lipofectamine 2000, according to the manufacturer's instructions. The transfected Top10 cells were cultured in ampicillin plates for 16 h. Positive cell clones were selected and cultured overnight. The recombinant plasmid was amplified in the Top10 competent cells. Restriction analysis with BamHI and NotI enzymes and DNA sequencing were used to confirm the true cloning of DLC-3.

Immunohistochemistry (IHC)

Briefly, tissue samples were fixed in formalin and embedded in paraffin. Sections were dewaxed in xylene and rehydrated through graded alcohols and water, and endogenous peroxidases were inactivated with 3% hydrogen peroxide in PBS, followed by incubation with the primary antibody overnight at 4°C and with the biotinylated secondary antibody at room temperature for 1 h. Omission of the primary antibody was used as a negative control. Then, the sections were detected with a streptavidin-peroxidase complex. Scoring was conducted according to the ratio and intensity of positive-staining cells: 0-5% scored 0; 6-35% scored 1; 36-70% scored 2; more than 70% scored 3. The final score of DLC-3 expression was designated as low or high expression group as follows: low expression: score 0-1; high expression: score 2-3. All the scores of DLC-3 expression were done in a blinded manner and determined independently by two senior pathologists.

Western blotting

Tissues or cells were lysed in RIPA buffer supplemented with protease inhibitor mixture for 30 min. at 4°C. The cell lysates were then sonicated briefly and centrifuged (14,000 × g at 4°C) for 15 min. to remove insoluble materials. Equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk and then incubated with first antibody, followed a horseradish peroxidase-conjugated secondary antibody.

Colony formation assay

Cells in the exponential phase of growth were selected and suspended into single cells by pipetting, and then inoculated into 10-cm Petri dishes. The cell suspension was further diluted with a gradient factor. Approximately 500 cells

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Table 1. Distribution of DLC-3 status in TNBC according to clinicopathologic characteristics

Clinicopathologic variables	Cases n = 100	DLC-3 expression level		P value
		No. of low expression	No. of high expression	
Age (years)				0.136
≤ 50	35	12	23	
> 50	69	20	45	
Tumor size (cm)				0.930
≤ 2	40	13	27	
> 2	60	19	41	
Differentiation grade				0.016*
Well-moderate	79	30	49	
Poor-undifferentiated	21	2	19	
Ki-67				0.213
≤ 20%	59	20	39	
> 20%	41	12	29	
Vascular invasion				0.647
Positive	28	8	20	
Negative	72	24	48	
Lymph node status				0.047*
Positive	55	13	42	
Negative	45	19	26	
TNM stage				0.539
I-II	86	29	57	
III	14	3	11	

*P < 0.05.

were added to the Petri dishes which were incubated at 37°C for 2-3 weeks until visible colonies appeared. Petri dishes were gently washed twice with PBS. Colonies were fixed with 5 ml methanol for 15 min, stained with Giemsa for 10-30 min, and then counted. Viable colonies containing at least 50 cells were counted.

MTT proliferation assay

The capability of cellular proliferation was measured by the [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] MTT assay. Approximately 5×10^3 cells were seeded into 96-well culture plates, then cells were incubated with 20 μ l MTT (10 mg/ml) for 4 h at 37°C and 200 μ l DMSO was pipetted to solubilize the formazan product for 20 min. at room temperature. The optical density was determined using a spectrophotometer at a wavelength of 570 nm.

Wound healing assay

A wound healing assay was performed to examine cell migration. Briefly, after the cells grew to

85% confluence in six-well plates, Wound was created by scraping with a pipette tip and the debris was washed with PBS. Photographs were taken at indicated time-points to assess the ability of the cells to migrate into the wound area. The scratch wounds were photographed using a Nikon inverted microscope with an attached digital camera, and the widths of the wounds were quantified using Image software.

Invasion assay

Chambers (Millipore, Billerica, MA, USA) with or without matrigel (BD Biosciences, San Jose, CA, USA) bedding were placed in 24-well plates. Cells (1×10^4) that were starved overnight were added to the upper chamber in 200 μ l of serum-free medium, respectively, while 500 μ l of complete medium to the bottom chamber.

After 24 h of incubation, cells in the upper chamber were fixed with 95% ethanol and stained for

30 min. with 4 g/L crystal violet. Cells on the underside of the chambers were counted under a 200 \times microscope field after the topside of the filter was wiped. Three independent invasion assays were performed in triplicate. Five random fields on average were counted using a light microscope.

Statistical analysis

Statistical analysis was carried out with the SPSS17.0 (San Rafael, CA, USA) software package for Windows. Comparison of categorical data was performed using the Chi-square test (χ^2 test) a comparison of continuous variables was performed using the Mann-Whitney U test. P < 0.05 was considered significant.

Results

Patients' characteristics

The profiles of the 100 patients and the tumor characteristics are shown in **Table 1**. All patients were female between the ages of 23 and 72 years (47.3 ± 9.5 years). According to

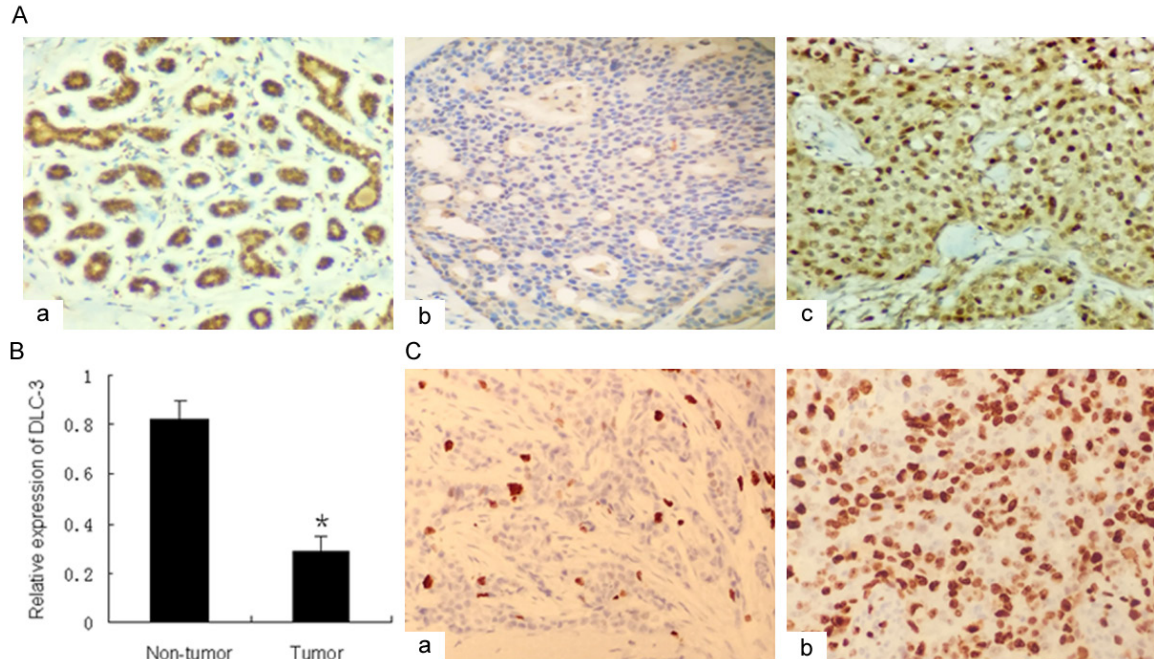


Figure 1. DLC-3 expression was frequently down-regulated in TNBC. (A) IHC results of DLC-3 expression in adjacent noncancerous and breast cancer tissue samples. DLC-3 was expressed in the nucleus of normal breast epithelial cells (Adjacent noncancerous) (a). Loss of expression of DLC-3 in poorly differentiated TNBC (b) and weak staining in well-differentiated TNBC (c). (B) Compared with normal breast tissue, DLC-3 expression was significantly decreased in TNBC (* $P < 0.05$). (C) IHC results of Ki-67 expression in breast cancer samples. (a) represents the low expression level group, (b) represents the high expression level group ($\times 200$).

the histologic grading, 79.0% (79/100) and 21.0% (21/100) of tumors were well-moderate, and poor-undifferentiation, respectively. Tumor size ranged from 0.5 to 8.2 cm (2.75 ± 1.23 cm). Tumors larger than 2 cm were present in 60.0% (60/100) of patients. Lymph node involvement was evident in 55.0% (55/100) of patients.

Expression of DLC-3 protein in TNBC

As shown in **Figure 1**, DLC-3 expressed in the nucleus of normal ductal epithelium and terminal duct-lobular unit of the breast, DLC-3 expression was reduced in poorly differentiated TNBC samples with syncytial growth pattern. Low DLC-3 protein expression was observed in 68.0% (68/100) of TNBC. Moreover, in order to further investigate the correlation between expression of DLC-3 and clinicopathologic features, samples were used for examination with IHC staining. Statistical analysis revealed negative DLC-3 expression was significantly associated with lymph node metastasis and histologic grade compared with those patients with positive DLC-3 expression (**Table 1**).

DLC-3 overexpression inhibits proliferation and colony formation of MDA-MB-231 cells

The expression of DLC-3 was downregulated in HCC-1937, MDA-MB-468 and MDA-MB-231 cells compared with the breast epithelial cell line MCF-10A. The lowest level of DLC-3 expression was found in MDA-MB-231 (**Figure 2**). Absorbance of transfected cells was detected every 24 h, from 24 to 72 h after transfection. Cell proliferation activity of the pcDNA3.1(+)-DLC-3 group was gradually inhibited from the second day after transfection, and as time passed, the cell proliferation activity of this group was significantly decreased compared with the pcDNA3.1(+) group and the untransfected MDA-MB-231 group. In addition, there was no significant difference between the proliferation of the untransfected MDA-MB-231 group and the pcDNA3.1(+) group, which suggested that DLC-3 overexpression may inhibit the proliferation of colon cancer cells. Moreover, a significant decrease was observed in the colony formation of the pcDNA3.1(+)-DLC-3 group when compared to the pcDNA3.1(+) group and the untransfected MDA-MB-231 group (**Figure 3**).

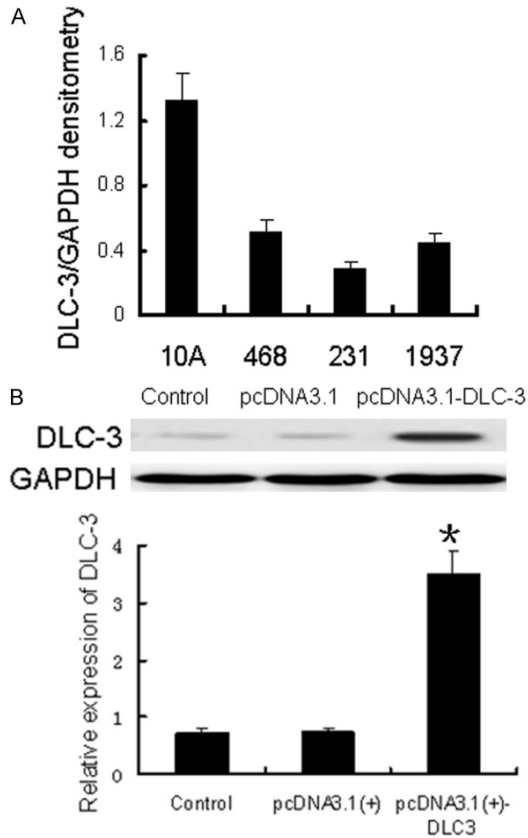


Figure 2. Effects of pcDNA3.1(+)-DLC-3 on DLC-3 expression in MDA-MB-231. A. The expression of DLC-3 in HCC-1937, MDA-MB-468, MDA-MB-231 and MCF-10A was detected by western blotting and normalized to GAPDH expression. B. DLC-3 protein expression in the pcDNA3.1(+)-DLC-3 cells was higher than that in the pcDNA3.1(+) group and the untransfected MDA-MB-231 group (* $P < 0.05$). No difference was observed between the pcDNA3.1(+) group and the untransfected MDA-MB-231 group.

Upregulation of DLC-3 suppresses the invasive ability of breast cancer cells

As wound healing assays suggested, compared to the control group, upregulation of DLC-3 significantly inhibited MDA-MB-231 cell migration in vitro. To further explore whether the reactivation of DLC-3 expression can regulate breast cancer invasion, we analyzed the invasion capability of the highly metastatic MDA-MB-231 cells using the methods described above. The number of MDA-MB-231 cells in the untreated group that migrated through the membrane was $(63.80 \pm 7.05)/HP$. A significant reduction of the number of invasive cells was observed at 24 h when cells infected with DLC-3 vector compared to the control. The number of invad-

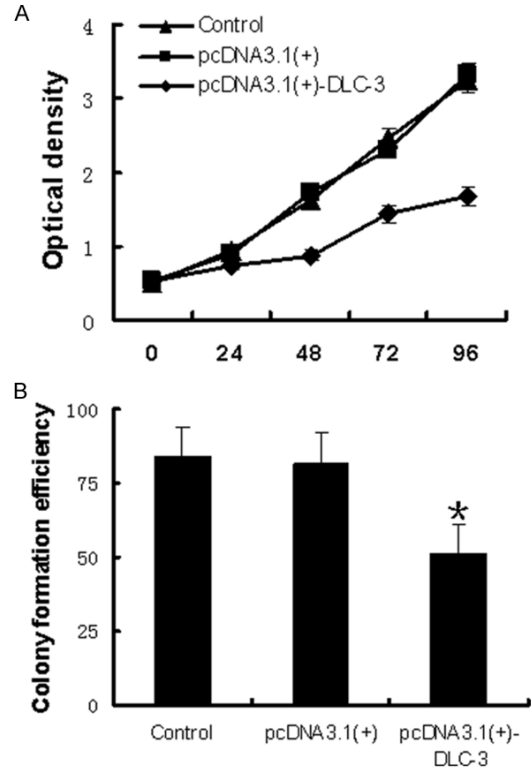


Figure 3. DLC-3 overexpression inhibits proliferation and colony formation of MDA-MB-231 cells. A. Cell proliferation of the pcDNA3.1(+)-DLC-3 cells was decreased compared with the pcDNA3.1(+) group and the untransfected MDA-MB-231 group. B. The colony formation of pcDNA3.1(+)-DLC-3 cells was decreased compared with the pcDNA3.1(+) group and the untransfected MDA-MB-231 group (* $P < 0.05$).

ed cells was significantly decreased when MDA-MB-231 cells infected with DLC-3 vector (37.21 ± 6.27)/HP (Figure 4).

DLC-3 overexpression regulates the Wnt/ β -catenin signaling pathway

The results of the Real-time PCR and western blot analyses showed that the expression of GSK-3 β protein was significantly higher than that in the pcDNA3.1(+) group and the MDA-MB-231 group. The level of β -catenin protein expression level was significantly lower than that in the pcDNA3.1(+) group and the MDA-MB-231 group. The c-myc protein expression level was significantly lower than that in the pcDNA3.1(+) group and the MDA-MB-231 group. Therefore, DLC-3 overexpression upregulates GSK-3 β , and downregulates mRNA and protein expression of β -catenin and c-myc (Figure 5).

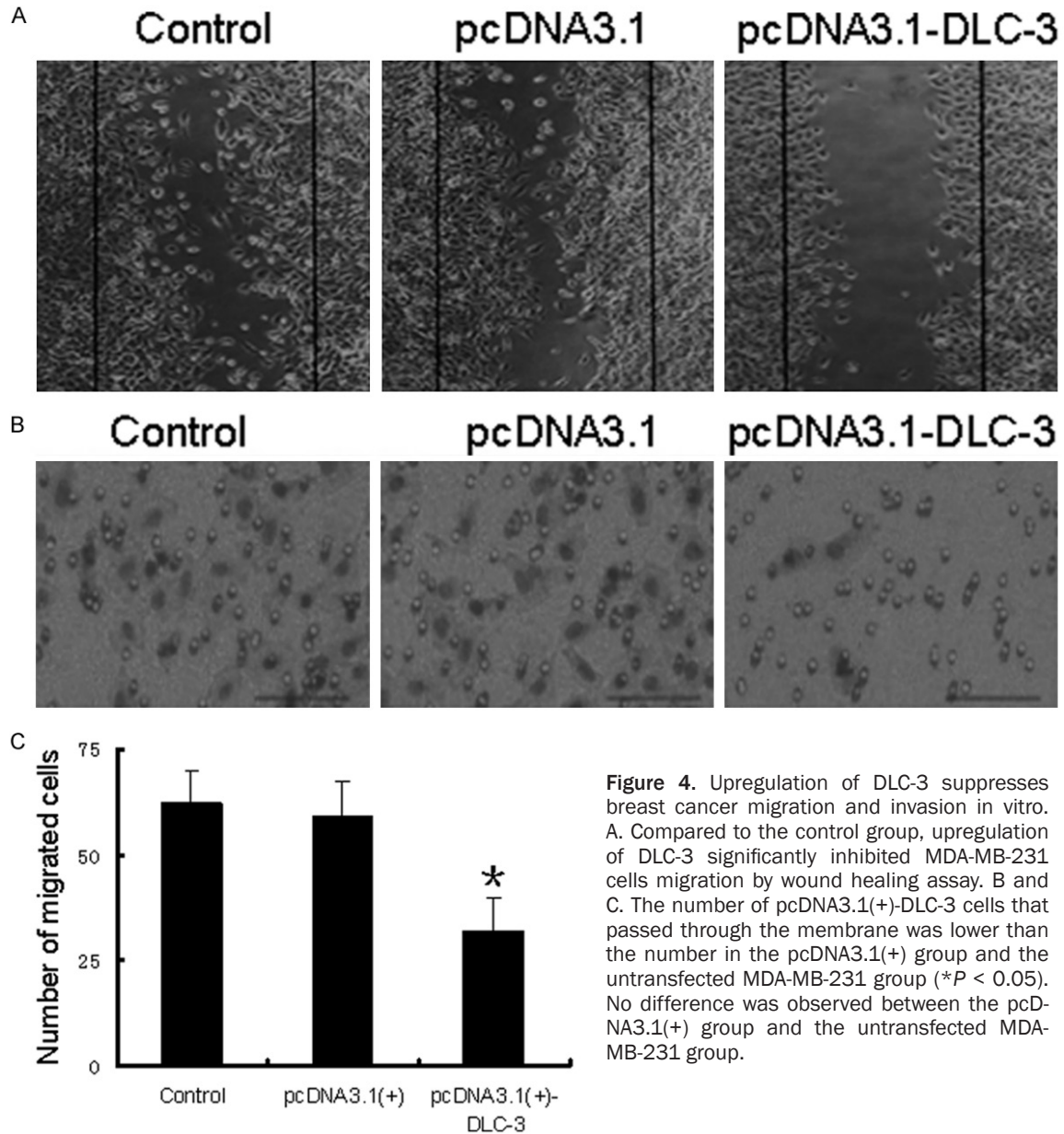


Figure 4. Upregulation of DLC-3 suppresses breast cancer migration and invasion in vitro. A. Compared to the control group, upregulation of DLC-3 significantly inhibited MDA-MB-231 cells migration by wound healing assay. B and C. The number of pcDNA3.1(+)-DLC-3 cells that passed through the membrane was lower than the number in the pcDNA3.1(+) group and the untransfected MDA-MB-231 group (* $P < 0.05$). No difference was observed between the pcDNA3.1(+) group and the untransfected MDA-MB-231 group.

Discussion

Decades of intense studies about Rho-GAPs had confirmed that Rho-GAP involved in the regulation of diverse cellular functions and closely correlated with multiple types of carcinomas [10]. It has been reported that deleted in liver cancer genes (DLC1-3) encoding Rho-GAPs were frequently downregulated or silenced in several human malignancies. The great similarity among the GAP domains of DLC-3 and those of DLC-1 and DLC-2 implied that DLC-3 may also act as a Rho-GAP that is essential for cell proliferation [11]. To date, studies have shown that DLC-3 downregulated in sev-

eral different types of cancers, including gastric cancer, prostate carcinoma, ovarian neoplasms and contributed to the process of tumorigenesis, whereas its overexpression could inhibit cell proliferation, colony formation [6, 8, 12]. Mokarram et al. found that the downregulated expression of DLC-3 in colorectal cancer may be due to DLC-3 gene promoter methylation [13]. To date, the role and underlying molecular mechanisms of DLC-3 in TNBC remain elusive. In this study, we demonstrated that DLC-3 was significantly downregulated in TNBC tissues. We also found that DLC-3 expression levels are associated with lymph node status and histological grade in TNBC. In order to

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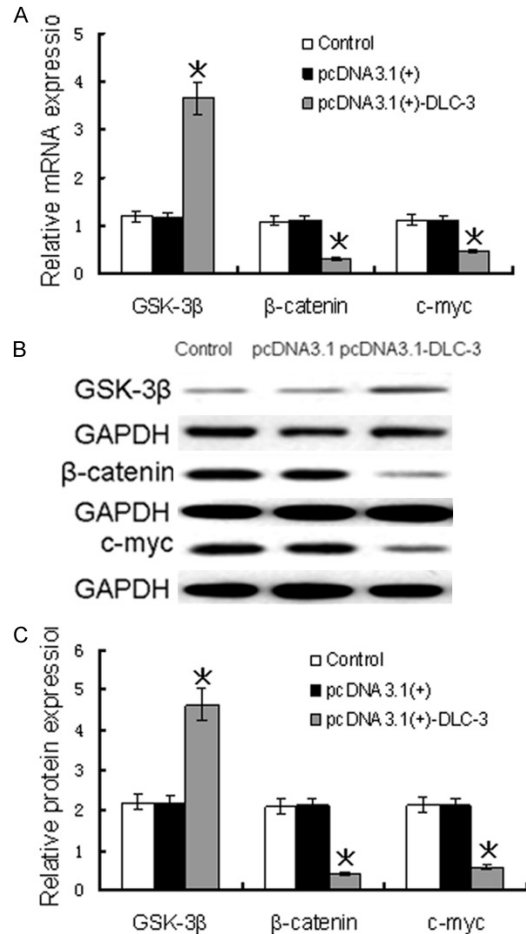


Figure 5. DLC-3 overexpression regulates the Wnt/ β -catenin signaling pathway. A. GSK-3 β mRNA expression was higher in the pcDNA3.1(+)-DLC-3 group, while β -catenin and c-myc mRNA expression was lower when compared to these levels in the pcDNA3.1(+) group and the untransfected MDA-MB-231 cells. B, C. GSK-3 β protein expression was higher in the pcDNA3.1(+)-DLC-3 group, while β -catenin and c-myc protein expression were lower, when compared to these levels in the pcDNA3.1(+) group and the untransfected MDA-MB-231 cells (* $P < 0.05$).

examine the role of DLC-3 in TNBC cell growth and invasion, we constructed a recombinant lentiviral vector coding DLC-3 and transfected the MDA-MB-231. Using wound healing and transwell assays, we revealed that upregulation of DLC-3 in MDA-MB-231 cells suppressed the migration and invasion of these TNBC. Therefore, the results of the present study demonstrated that DLC-3 may act as a tumor-suppressor gene in TNBC. However, although the role of DLC-3 during the pathogenesis of TNBC has been suggested, the detailed molecular pathway and downstream targets have not yet been fully elucidated.

Research suggested that the tumor suppressor DLC-1 relate to the expression of cyclin D1 [14, 15]. Cyclin D1 is the target gene of the Wnt/ β -catenin signaling pathway, and it is tightly related to β -catenin. The Wnt signaling pathway is highly conserved in various species, controlling cell proliferation, cell polarity, and cell fate among other activities [16, 17]. In addition, it has been shown that it is often dysregulated in a variety of diseases including cancer. Extensive studies have shown that the Wnt/ β -catenin signaling pathway plays important roles in breast cancer [18, 19]. The Wnt signaling pathway consists of several key components such as Wnt proteins, β -catenin, GSK-3 β and APC protein. In normal intestinal mucosa, APC expression increases gradually from the bottom to the top of intestinal crypts. When the Wnt signaling pathway is inactive, a destruction complex consisting of APC, Axin, and GSK-3 β promotes β -catenin degradation, which causes a relatively low β -catenin protein level in intestinal cancer cells [20, 21]. We analyzed the expression levels of the Wnt pathway components GSK-3 β , β -catenin and c-myc gene by real-time PCR and western blot. These results showed that DLC-3 overexpression suppressed the Wnt/ β -catenin signaling pathway by upregulating GSK-3 β , downregulating β -catenin and c-myc. The results suggested that DLC-3 interacted with the Wnt/ β -catenin signaling pathway. However, the exact details of this process are still unknown. Therefore, in forthcoming studies, we will elucidate the relationship between DLC-3 and the Wnt/ β -catenin signaling pathway in TNBC.

In conclusion, we revealed that DLC-3 was downregulated in TNBC, and its expression level was associated with lymph node status and histologic grade in breast cancer. Furthermore, with gene transfection technology, we showed that upregulation of DLC-3 expression could suppress breast cancer cell growth and invasion in vitro. These data provided a sound scientific rationale for further investigation into targeting DLC-3 in TNBC.

Acknowledgements

This study was supported by Qingdao Scientific and Technologic Development Program (No. 16-6-2-15-nsh), National Natural Science Foundation of China (No. 81700029), Natural Science Foundation of Shandong Province (No.

ZR2017MH017) and Higher Educational Science and Technology Program of Shandong Province (No. J17B092).

Disclosure of conflict of interest

None.

Address correspondence to: Zhi-Dong Lv, Center of Diagnosis and Treatment of Breast Disease, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, P. R. China. Tel: +86 053282913059; Fax: +86 053282913536; E-mail: zhidonglv@126.com; Zhao-Chuan Yang, Department of Child Health Care, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, P. R. China. Tel: +86 053282913057; E-mail: yzhchuanchuan@163.com

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