Long non-coding RNA DGCR5 incudes tumorigenesis of triple-negative breast cancer by affecting Wnt/β-catenin signaling pathway

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Summary

Purpose: Triple-negative breast cancer (TNBC) is one of the most ordinary malignant tumors. Recent studies have revealed that long noncoding RNAs (lncRNAs) play an important role in the progression of tumorigenesis. This study aimed to identify how lncRNA DGCR5 functions in the progression of TNBC.

Methods: DGCR5 expression of both 57 paired TNBC patients' tissue samples and cells was detected by real-time quantitative polymerase chain reaction (RT-qPCR). Moreover, the function of SNHG7 was identified by performing proliferation assay and transwell assay in vitro. Besides, the underlying mechanism was explored through Western blot assay and RT-qPCR. In addition, tumor formation and metastasis assays were also conducted in vivo.

Results: In this study, DGCR5 expression was obviously higher in TNBC tissues when compared with that in adjacent non-tumor samples. Cell proliferation, migration and invasion in TNBC were inhibited after knockdown of DGCR5 in vitro. Moreover, results of further experiments revealed that the targeted proteins in Wnt/β-catenin signaling pathway were downregulated via knockdown of DGCR5 in TNBC. Furthermore, tumor formation and metastasis of TNBC were inhibited via knockdown of DGCR5 in nude mice.

Conclusions: Our study suggests that DGCR5 enhances TNBC cell proliferation and metastasis via inducing Wnt/β-catenin signaling pathway in vitro and in vivo.

Key words: long noncoding RNA, DGCR5, triple-negative breast cancer, Wnt/β-catenin signaling pathway

Introduction

Breast cancer is one of the most common malignancies diagnosed in women which can be classified into several histological types. Triple-negative breast cancer (TNBC), defined as lack of expression of the progesterone receptor (PR), the estrogen receptor (ER), and the human epidermal growth factor receptor 2 (HER2), is the most aggressive type of breast cancer with poor prognosis. TNBC represents 8–15% of all breast cancer cases and is characterized by high grade, high proliferation, age <38 years, lymph node or distant metastases and lack of BRCA1 protein expression [1,2]. Despite the advances made in diagnostic tools and treatments, the morbidity and mortality of TNBC remain unsatisfactory [3]. Thus, it is urgent to explore the molecular mechanism underlying TNBC and improve the poor prognosis of these patients.

Long non-coding RNAs (lncRNAs) are known as a cluster of non-coding transcripts which have been reported to play an important role in the regulation of various biological processes, including cell proliferation, differentiation and apoptosis. For

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example, lncRNA SNHG5 serves as an important anti-oncogene in the progression of gastric cancer through trapping MTA2 in the cytosol [4]. LncRNA ZEB1-AS1 acts as an oncogene in prostate cancer by activating ZEB1 and regulating the downstream molecules of ZEB1 [5]. LncRNA H19 promotes cell proliferation and cell migration in pancreatic cancer which is modulated by miR-194 [6]. LncRNA FALEC facilitates cell proliferation in melanoma by silencing p21, which is associated with poor prognosis of patients with melanoma [7].

Our study demonstrated that DGCR5 was remarkably upregulated in TNBC tissues and cell lines. Moreover, knockdown of DGCR5 inhibited the proliferation, migration and invasion of TNBC *in vitro* and *in vivo*. In addition, we further found that the function of DGCR5 in TNBC was also associated with the activation of Wnt/β-catenin signaling pathway.

Methods

Tissue specimens

57 paired tumor tissues and adjacent non-tumor tissues were sequentially gathered from TNBC patients who underwent surgery at our hospital. All cases were diagnosed with TNBC by two independent pathologists0 without any controversy.

Cell culture

Human BC cells (MCF-7, LCC9, T-47D, SKBR3) and normal human breast cell line (MCF-10A) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Culture medium consisted of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM) as well as 100 U/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA). Besides, cells were cultured in an incubator containing 5% CO_2 at 37°C.

Cell transfection

Specific short-hairpin RNA (shRNA; BiosettiaInc., San Diego, CA, USA) against DGCR5 was synthesized. Negative control (NC) shRNA was also synthesized. DGCR5 shRNA (sh-DGCR5) and NC were then used for transfection in T-47D BC cells. 48 h later, real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect transfection efficiency in these cells.

RNA extraction and RT-qPCR

Total RNA was extracted from cultured TNBC cells or patients' tumor tissues by using TRIzol reagent (TaKaRa Bio, Inc., Shiga, Japan) and then reverse-transcribed to cDNAs through reverse Transcription Kit (TaKaRa, Shiga, Japan). The primer sequences used for RT-qPCR were as follows: DGCR5 forward: 5'-CACACGCATGGAAAACCCAG-3' and reverse: 5'-GAGGACCTGAGCTGTAAGCC-3'; Glyceraldheyde 3-phosphate dehydrogenase (GAPDH),

forward: 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse: 5'-TGGTGAAGACGCCAGTGGA -3'. PCR was performed three times in the following sequence: pre-denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. $2^{-\Delta\Delta Ct}$ method was utilized for calculating relative expression.

Cell proliferation assay

Before transfection, cells (1000 cells/well) were seeded into 96-well plates for 12 h. After they were cultured at different times (0, 24, 48, and 72 h), 15 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO, USA) were added to each well and incubated for 4 h. To stop the reaction, they were added with 100 μL dimethyl sulfoxide (DMSO). Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Transwell assay

$2×10^4$ cells in serum-free DMEM were replanted in the upper chamber which were coated with 30 μL of Matrigel (BD Biosciences, San Jose, CA, USA). DMEM and fetal bovine serum (FBS) were added into the bottom chamber. After 24 h of incubation, the cells were immersed with 4% paraformaldehyde for 10 min and stained in 1% crystal violet for 30 min to remove any uninfected cells from the upper chamber. Next, cells were counted and photographed in randomly selected fields with a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

Western blot analysis

Cell samples were washed with precooled phosphate buffered saline (PBS) and then lysed with cell lysis solution (RIPA) (Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA) (Thermo Fisher Scientific Inc., Waltham, MA, USA). The proteins were transferred on to a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland), blocked in tris buffered saline-tween (TBST) (25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of target proteins including Wnt3a, β-catenin, C-myc and Survivin (Abcam Inc., Cambridge, MA, USA) in Wnt/β-catenin signaling pathway and GAPDH (Abcam Inc., Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3×10 min) with TBST, the secondary antibody was added and incubated at room temperature for 1 h. The results were analyzed by Image J software (NIH, Bethesda, MD, USA).

Xenograft model

For the tumor formation assay, transfected cells were subcutaneously injected into NOD/SCID mice (6 weeks old). Tumor diameters were detected every 5 days after inoculation. Tumor volume was calculated according to the formula: (volume= length×width^2×1/2). Tumors were extracted after 4 weeks after the mice were euthanized. For the tumor metastasis assay, transfected

cells were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed, and the lungs were extracted after 4 weeks. Then the number of metastatic nodules in the lung was counted.

Statistics

All statistical analyses were performed by SPSS 21.0 software package (IBM Corp., Armonk, NY, USA). Independent-sample t-test was used to compare the difference between two groups and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression level of DGCR5 in tissues and cells of TNBC

RT-qPCR was conducted for detecting DGCR5 expression in 57 patients' tissues and 4 TNBC cell lines. DGCR5 was significantly upregulated in tu-

mor tissue samples than that in adjacent tissues (Figure 1A). Analysis of clinicopathological features in those patients demonstrated that downregulated DGCR5 was obviously correlated to lymph node metastasis and tumor stage (Table 1). Compared with the expression in MCF-10A, DGCR5 level was significantly higher in TNBC cells (Figure 1B).

Knockdown of DGCR5 repressed cell proliferation and metastasis in T-47D TNBC cells

In our study, we chose T-47D cell line for the knockdown of DGCR5. Then RT-qPCR was utilized for detecting the DGCR5 expression (Figure 2A). Moreover, MTT assay showed that the cell growth ability was significantly repressed after DGCR5 was knocked down (Figure 2B). Transwell assay showed that the number of migrated cells was significantly
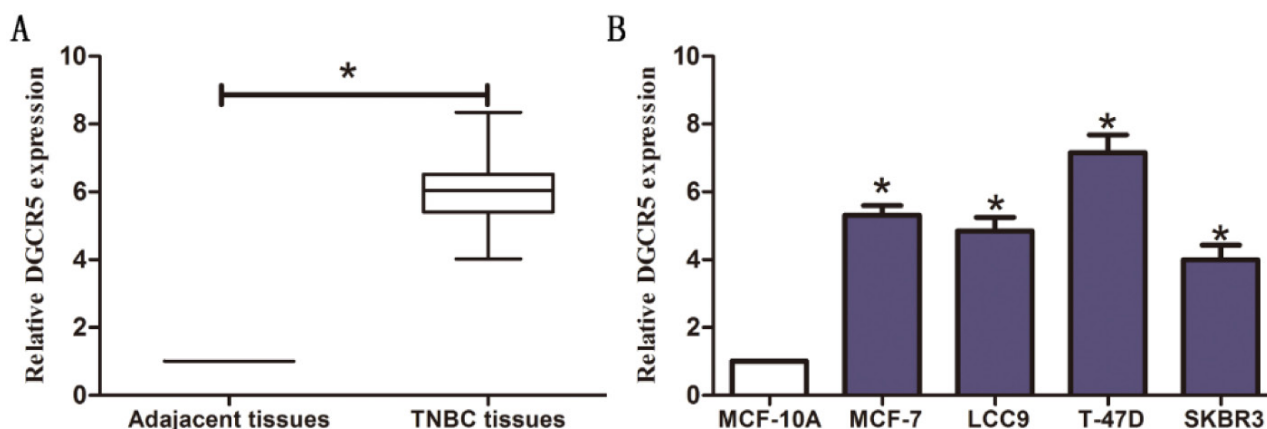
Figure 1. Expression level of DGCR5 was increased in TNBC tissues and cell lines. **A:** DGCR5 expression was significantly increased in the TNBC tissues compared with adjacent tissues. **B:** Expression levels of DGCR5 relative to GAPDH were determined in the human TNBC cell lines and MCF-10A by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p < 0.05$.

**Table 1.** Correlation between lncRNA DGCR5 expression and clinicopathological characteristics in TNBC patients

Characteristics	Patients	Expression of lncRNA DGCR5		p value
		Low-DGCR5	High-DGCR5	
Total	57	26	31	
Age (years)				0.749
≤50	25	12	13	
>50	32	14	18	
TNM stage				0.018
I-II	32	19	13	
III-IV	25	7	18	
Tumor size (cm)				0.866
<3	30	14	16	
>3	27	12	15	
Lymph node metastasis				0.027
No	26	16	10	
Yes	31	10	21	

decreased after DGCR5 was knocked down (Figure 2C). Furthermore, transwell assay showed that the number of invaded cells was significantly reduced after DGCR5 was knocked down (Figure 2D).
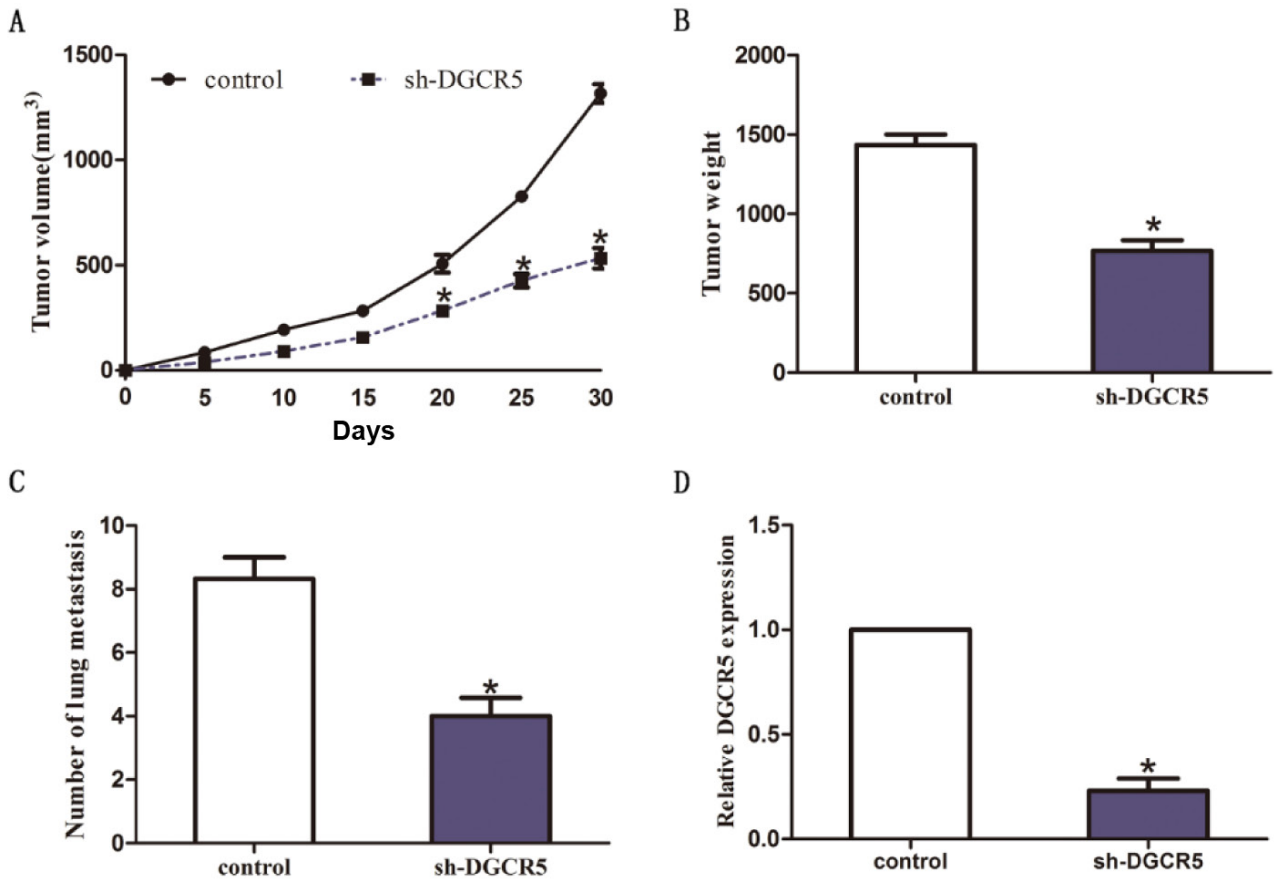
DGCR5 knockdown inhibited tumor formation and metastasis in vivo

Then, the ability of DGCR5 in tumor formation and metastasis was detected *in vivo*. The tumor size in sh-DGCR5 group was smaller compared with negative control shRNA group (Figure 3A). The weight of dissected tumors in sh-DGCR5 group was smaller compared with negative control shRNA group (Figure 3B). The number of metastatic nodules in the lung from the sh-DGCR5 group was significantly reduced compared to negative control shRNA group (Figure 3C). Moreover, the expression level of DGCR5 in dissected tumor tissues was detected by RT-qPCR and the results showed that DGCR5 was lower-expressed in sh-DGCR5 group compared with negative control shRNA group (Figure 3D).
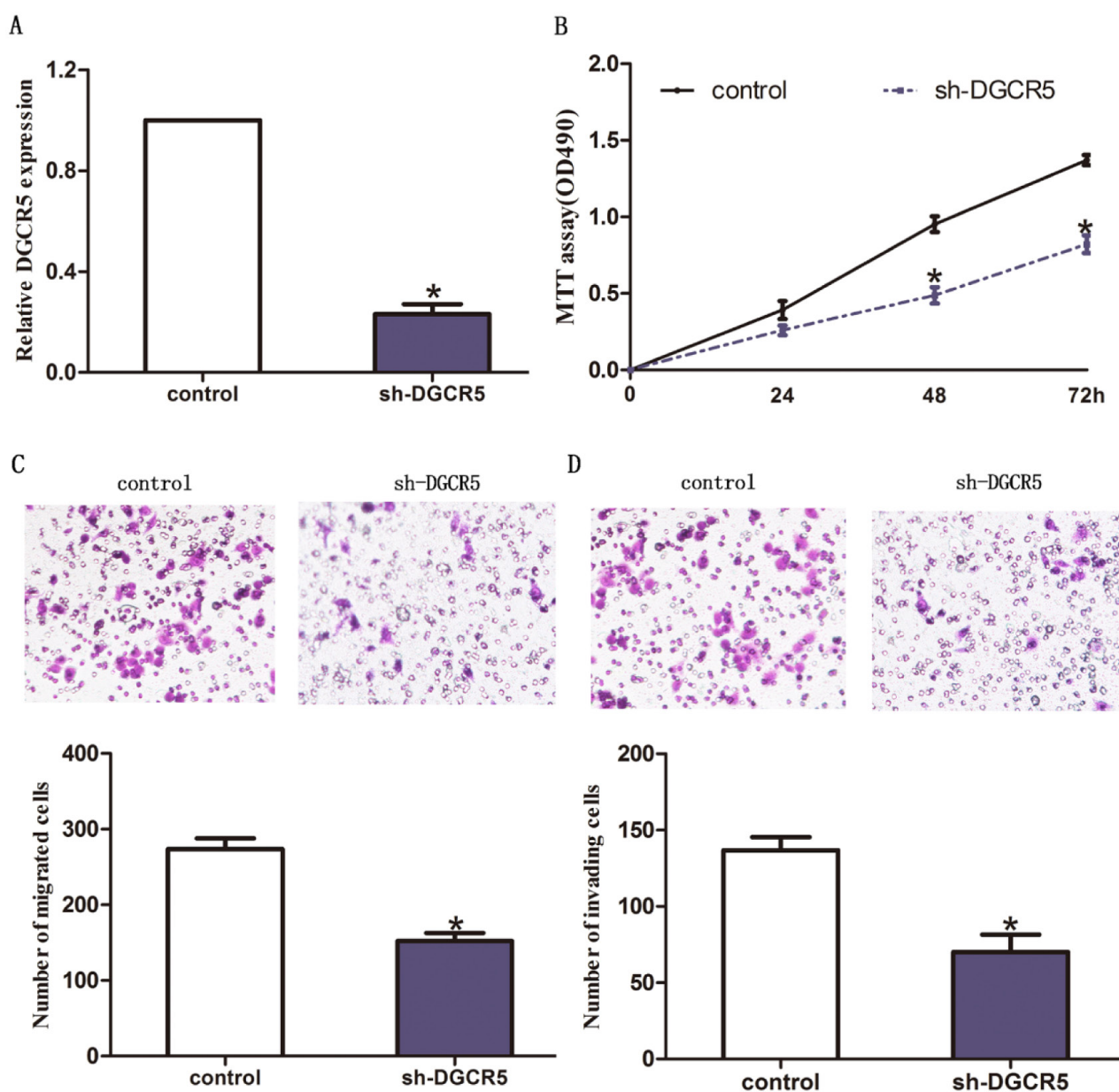
Figure 2. Knockdown of DGCR5 inhibited T-47D cell proliferation, migration and invasion. **A:** DGCR5 expression in TNBC cells transfected with negative control shRNA (NC) or DGCR5 shRNA (sh-DGCR5) was detected by RT-qPCR. GAPDH was used as an internal control. **B:** MTT assay showed that knockdown of DGCR5 significantly repressed cell proliferation in TNBC cells. **C:** Transwell assay showed that knockdown of DGCR5 significantly repressed cell migration in TNBC cells (magnification: 40×). **D:** Transwell assay showed that knockdown of DGCR5 significantly repressed cell invasion in TNBC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

Figure 3. Knockdown of DGCR5 inhibited tumor formation and metastasis of TNBC *in vivo*. **A:** The tumor size in sh-DGCR5 group was smaller compared with NC group. **B:** The weight of dissected tumors in sh-DGCR5 group was smaller compared with NC group. **C:** The number of metastatic nodules in the lung from the sh-DGCR5 group was significantly reduced compared to NC group. **D:** DGCR5 of those dissected tumors was lower-expressed in sh-DGCR5 group compared with NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.
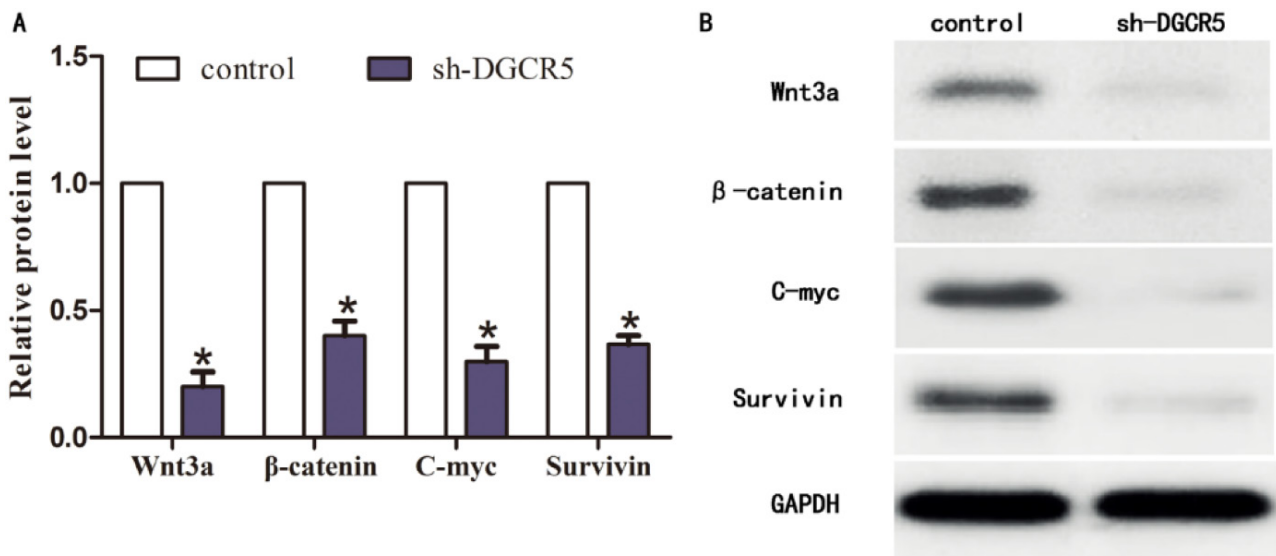
Figure 4. The association between DGCR5 and Wnt/β-catenin signaling pathway in TNBC. **A:** RT-qPCR results revealed that the expression of target proteins in Wnt/β-catenin signaling pathway was downregulated in sh-DGCR5 group compared with NC group. **B:** Western blot assay results revealed that the expression of target proteins in Wnt/β-catenin signaling pathway was downregulated in sh-DGCR5 group compared with NC group. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05.

The interaction between Wnt/β-catenin signaling pathway and DGCR5 in TNBC

To explore the underlying mechanism of DGCR5 function in TNBC, RT-qPCR and Western blot assay were conducted to detect the target proteins in Wnt/β-catenin signaling pathway such as Wnt3a, β-catenin, C-myc and Survivin. RT-qPCR results showed that Wnt3a, β-catenin, C-myc and Survivin could be downregulated *via* knockdown of DGCR5 (Figure 4A). Western blot assay results showed that Wnt3a, β-catenin, C-myc and Survivin could be downregulated *via* knockdown of DGCR5 (Figure 4B). These results suggested that DGCR5 participated in the regulation of Wnt/β-catenin signaling pathway and further promoted TNBC development and metastasis.

Discussion

A large body of evidence has suggested that lncRNAs are crucial regulators in carcinogenesis of TNBC. For instance, lncRNA RMST inhibits cell proliferation, cell migration and promotes cell apoptosis in TNBC [8]. Through binding miR-196a-5p competitively, lncRNA GAS5 inhibits the progression of TNBC *via* inhibiting cell proliferation and invasion [9]. LncRNA MALAT1 enhances cell proliferation and cell invasion in TNBC through targeting miR-129-5p [10]. LncRNA snaR is over-expressed in TNBC and facilitates TNBC cell proliferation and cell migration [11].

DiGeorge syndrome critical region gene 5 (DGCR5) was first reported to be downregulated in Huntington's disease. Recently, DGCR5 is found to be a crucial regulator in the progression of many cancers, including cervical cancer, gastric cancer, bladder cancer, lung cancer and so on [12-15]. In the present study, DGCR5 was found to be upregulated in both TNBC tissues and cells. Significant correlation was seen between DGCR5 expression and tumor stage, lymph node metastasis. Furthermore, after DGCR5 was knocked down, the ability of cell growth, migration and invasion was suppressed.

These data indicated that DGCR5 functioned as an oncogene and promoted the tumorigenesis of TNBC.

Wnt proteins play an important role in the regulation of diverse processes during embryogenesis by modulating stem cell division and migration. Recently, more and more researches have revealed that aberrant activation of the Wnt/β-catenin signaling happens in the development of several human cancers. For instance, by activation of Wnt/β-catenin signaling inhibitors, DKK1 and SFRP2, TET1 serves as a tumor suppressor in ovarian cancer *via* inhibiting epithelial-mesenchymal transition (EMT) [16]. LncRNA CRNDE enhances cell proliferation and chemoresistance in colorectal cancer *via* modulating the expression of miR-181a-5p which directly mediates the regulation of Wnt/β-catenin pathway [17]. Through modulating Wnt/β-Catenin/Axin2 signaling, c-Myb facilitates cell invasion and cell migration in breast cancer [18].

In the present study, we detected the expression of Wnt3a, β-catenin, C-myc and Survivin, which were the target proteins in Wnt/β-catenin signaling pathway in TNBC cells after knockdown of DGCR5. The results showed that target proteins in Wnt/β-catenin signaling pathway could be downregulated *via* knockdown of DGCR5. All the results above suggested that DGCR5 might promote tumorigenesis of TNBC *via* activating Wnt/β-catenin signaling pathway.

Conclusions

To sum up, DGCR5 could enhance TNBC cell proliferation and metastasis through activating Wnt/β-catenin signaling pathway. These findings implied that lncRNA DGCR5 could serve as a promising marker for TNBC.

Conflict of interests

The authors declare no conflict of interests.

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