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DGCR8 promotes the metastasis in triple-negative breast cancer by epigenetically regulating TGF-β

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Abstract. – OBJECTIVE: Breast cancer (BC) is one of the most ordinary fatal cancers. Recent studies have identified the vital role of genes in the development and progression of Tri-negative breast cancer (TNBC). In this research, DGCR8 was studied to identify how it functioned in the metastasis of TNBC.

PATIENTS AND METHODS: DGCR8 expression of tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in 50 TNBC patients. Wound healing assay and transwell assay were used to observe the changes in the biological behaviors of TNBC cells through knockdown or overexpression of DGCR8. In addition, qRT-PCR and Western blot assay were performed to discover the potential target protein of DGCR8 in TNBC.

RESULTS: DGCR8 expression level in TNBC samples was higher than that of adjacent ones. Besides, the migration ability and invasion ability of TNBC cells were inhibited after DGCR8 was silenced, while they were promoted after DGCR8 was overexpressed. In addition, TGF-β was downregulated after silencing of DGCR8 in TNBC cells, while TGF-β was upregulated after overexpression of DGCR8 in TNBC cells. Furthermore, TGF-β was upregulated in TNBC tissues, which was positively associated with DGCR8.

CONCLUSIONS: Our study uncovers a new oncogene in TNBC and suggests that DGCR8 can enhance TNBC cell migration and invasion via targeting TGF-β, which provides a novel therapeutic target for TNBC patients.

Key Words:
DGCR8, Tri-negative breast cancer, TGF-β.

Introduction

Tri-negative breast cancer (TNBC) is a group of breast cancer with negative estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2). It is a highly invasive clinical subtype of breast cancer, accounting for about 15% of all pathological types. The prognosis of patients with TNBC is poor, with high recurrence, metastasis, and mortality rates[1]. Because of the specificity of TNBC gene expression, the targeted therapy cannot be used for intervention. Chemotherapy is the main treatment method. At present, chemotherapeutic drugs include taxanes (paclitaxel, docetaxel, albumin synthesis of paclitaxel), anthracyclines (adriamycin, epirubicin, lipoid adriamycin), anti-metabolites (capecitabine, gemcitabine) and so on[2]. So far, there is no standard treatment guideline for TNBC. Therefore, it is of great clinical significance to explore the therapeutic targets of TNBC.

DGCR8 is an allele located in the ql1.2 region of human chromosome 22. It is associated with DiGeorge Syndrome (DGCS), so it is named DGCR8[3]. DGCR8 is an RNA-binding protein involved in the synthesis of microRNAs. In the synthesis of microRNAs, it binds pri-microRNAs and promotes the cleavage of Drosha by interacting with Drosha and stabilizing microprocessors, which regulate the production of microRNAs and affect the proliferation, migration, and invasion of tumors[4,5]. The high expression of DGCR8 promotes the occurrence, development, and metastasis of cancer, including kidney clear cell carcinoma, thyroid carcinoma, and ovarian cancer[6-8]. Up to now, there are few reports about the protein in TNBC, and its function is still unclear.

Meanwhile, the clinical role and underlying mechanisms of DGCR8 in the development of TNBC remain unexplored. In the present study,

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we performed function and mechanism assays to explore whether DGCR8 functioned in the metastasis of TNBC.

Patients and Methods

Patients and Clinical Samples

Human tissues were harvested from 50 TNBC patients who received surgery at our Hospital. Before operation, written informed consent was achieved. No radiotherapy or chemotherapy was performed for any patients before the operation. Tissues got from the surgery were stored immediately at -80°C. This study was approved by the Ethics Committee of our hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human TNBC 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). The cells were cultured with medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), as well as 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in an incubator containing 5% CO_2 at 37˚C.

Cell Transfection

Specific short-hairpin RNA (shRNA; Biosettia Inc., San Diego, CA, USA) against DGCR8 was synthesized. Negative control shRNA was also synthesized. DGCR8 shRNA (sh-DGCR8) and negative control (control) were then used for transfection in T-47D TNBC cells. Besides, lentivirus (Biosettia Inc., San Diego, CA, USA) against DGCR8 (DGCR8) was synthesized, and then, used for transfection in MCF-7 TNBC cells. Empty vector was used as control. 48 h later, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect transfection efficiency in these cells.

RNA Extraction and RT-qPCR

The total RNA was separated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using the Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Following are the primers using for qRT-PCR: AK027294 primers forward: 5'-AACGGGAAATCCGAGGTCTG-3', reverse: 5'-GGCTCACTTGGGTTCTCACA-3'; GAPDH primers forward: 5'-CACCCACTCCTCCACCTTTG-3' and reverse 5'-CCACCACCCTGTTGCTGTAG-3'. Thermocycling conditions are as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 60°C for 30 s, for a total of 35 cycles.

Wound Healing Assay

1.0×10^4 cells were seed into a 6-well plate with cells in each well. Three parallel lines were made on the back of each well. After about 90% of cells were fused, they were scratched with a pipette tip and cultured in a medium, followed by photography under a light microscope (Olympus, Tokyo, Japan) after 0 and 48 h. Each assay was independently repeated in triplicate.

Transwell Assay

24 h after transfection, 2×10^5 cells in 100 μL serum-free DMEM were transformed to top chamber of an 8-μm culture inserts (Corning, Corning, NY, USA) coated with 50 μg Matrigel (BD Biosciences; Franklin Lakes, NJ, USA). 20% FBS-DMEM was added to the lower chamber of the culture inserts. 24 h later, these inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. An inverted microscope (×40) was utilized for counting invaded cells in three random fields.

Western Blot Analysis

The protein was extracted from cells using the radioimmunoprecipitation assay reagent (RIPA) (Beyotime, Shanghai, China), and its concentration was quantified using the TNBC protein assay kit (TaKaRa, Dalian, China) protein. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with antibodies after replaced to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Cell Signaling Technology (CST; Danvers, MA, USA) provided us rabbit anti-β-actin and rabbit anti-TGF-β, as well as goat anti-rabbit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for assessment of protein expression.

Statistical Analysis

All statistical analyses were performed by GraphPad Prism 5.0 (La Jolla, CA, USA). The difference between the two groups was compared by independent-sample *t*-test. The statistically significance was defined as $p<0.05$.

Results

DGCR8 Expression Level in TNBC Tissues and Cells

To determine the biological function of DGCR8 in the tumorigenesis of triple-negative breast cancer, we detected DGCR8 expression levels in 50 paired TNBC specimens by qRT-PCR. DGCR8 was significantly upregulated in TNBC tissue samples compared with adjacent tissues (Figure 1A). DGCR8 expression was also detected *via* qRT-PCR in four TNBC cell lines. DGCR8 expression level in TNBC cells was higher than that of MCF-10A (Figure 1B).

Knockdown of DGCR8 Suppressed Cell Migration and Invasion in TNBC Cells

To further investigate whether DGCR8 is connected to the metastasis of TNBC, we researched the function of DGCR8 *in vitro*. In this study, we chose T-47D TNBC cell line for the silence of DGCR8. Then, DGCR8 expression was detected by qRT-PCR (Figure 2A). In this research, the ability of cell migration was examined *via* wound healing assay after the knockdown of DGCR8 in the T-47D cells. The results revealed that the rate of T-47D cell migration was considerably reduced after the knockdown of DGCR8 (Figure 2B). The outcome of transwell assay also revealed that the number of invaded cells was remarkably decreased after DGCR8 was silenced in TNBC cells (Figure 2C).

Overexpression of DGCR8 Promoted Cell Migration and Invasion in TNBC Cells

MCF-7 TNBC cell line was chosen for overexpression of DGCR8. Then, DGCR8 expression was detected by qRT-PCR (Figure 3A). Moreover, the results of wound healing assay showed that the overexpression of DGCR8 significantly promoted the migration ability of TNBC cells (Figure 3B). The outcome of transwell assay also revealed that the number of invading cells was remarkably increased after DGCR8 was overexpressed in TNBC cells (Figure 3C).

The Interaction Between TGF-β and DGCR8 in TNBC

qRT-PCR results showed that TGF-β was downregulated in sh-DGCR8 group compared with the control group (Figure 4A). Meanwhile, TGF-β was upregulated in DGCR8 group compared with empty vector group (Figure 4B). Western blot assay results showed that TGF-β was downregulated in sh-DGCR8 group compared with control group (Figure 4C). Meanwhile, TGF-β was upregulated in DGCR8 group compared with empty vector group (Figure 4D). Furthermore, TGF-β expression of TNBC tissues was significantly higher than that of adjacent tis-

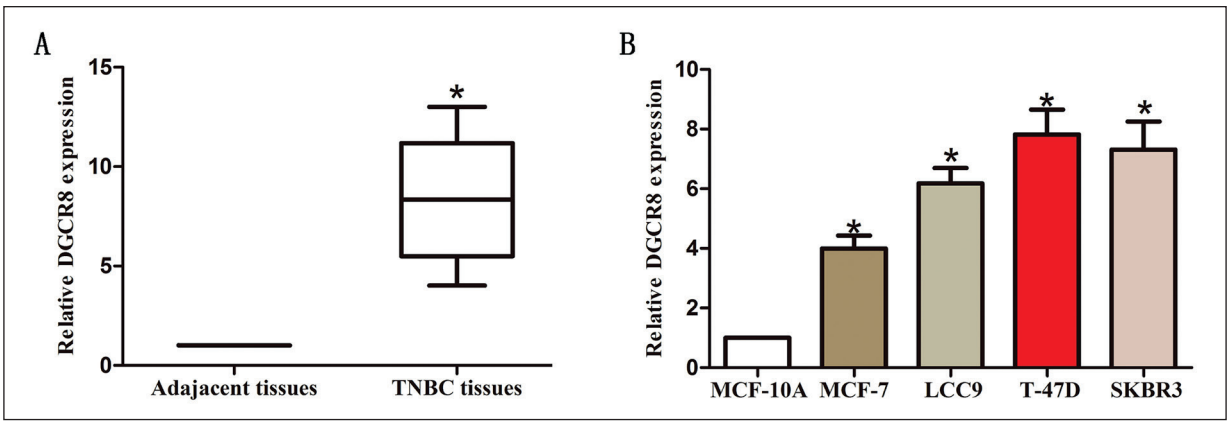

Figure 1. Expression level of DGCR8 was increased in TNBC tissues and cell lines. **A,** DGCR8 expression was significantly increased in the TNBC tissues compared with adjacent tissues. **B,** Expression levels of DGCR8 relative to β-actin were determined in the human TNBC cell lines and MCF-10A (normal human breast cell line) by qRT-PCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

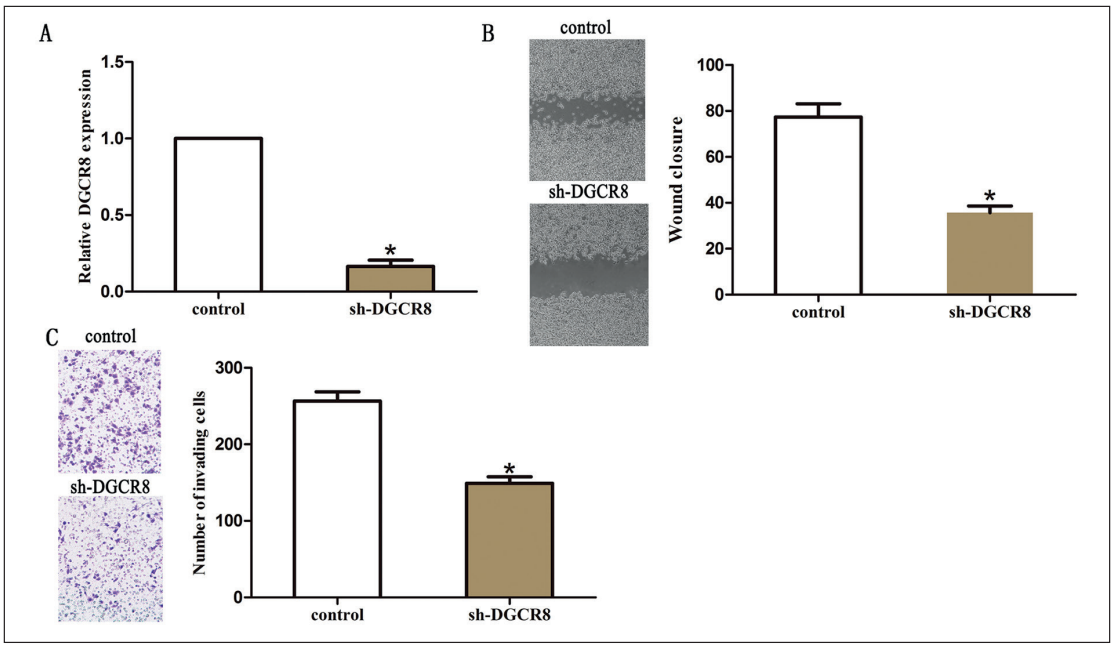

Figure 2. Knockdown of DGCR8 inhibited T-47D TNBC cell migration and invasion. **A,** DGCR8 expression in TNBC cells transduced with DGCR8 shRNA (sh-DGCR8) and the negative control (control) was detected by qRT-PCR. β-actin was used as an internal control. **B,** Wound healing assay showed that silence of DGCR8 significantly repressed cell migrated ability of TNBC cells (magnification: 40×). **C,** Transwell assay showed that number of invaded cells was significantly decreased *via* silence of DGCR8 in TNBC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

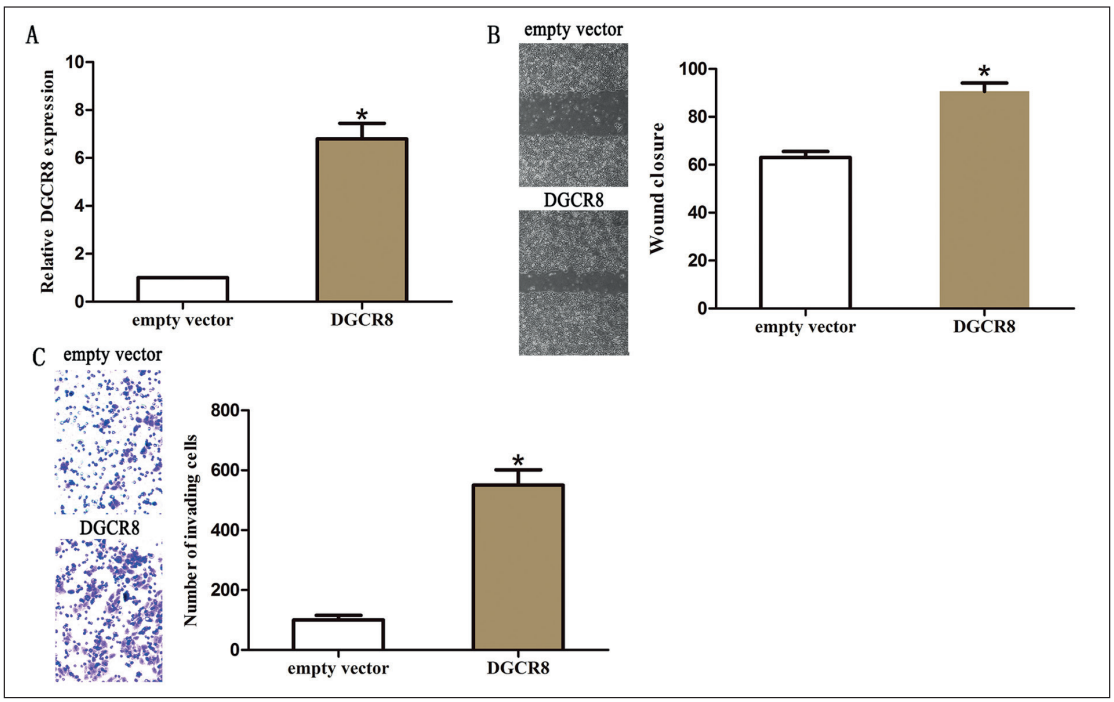

Figure 3. Overexpression of DGCR8 promoted MCF-7 TNBC cell proliferation and invasion. **A,** DGCR8 expression in TNBC cells transduced with DGCR8 lentivirus (DGCR8) and the empty vector was detected by RT-qPCR. β-actin was used as an internal control. **B,** Wound healing assay showed that overexpression of DGCR8 significantly promoted cell migrated ability of TNBC cells (magnification: 40×). **C,** Transwell assay showed that number of invaded cells was significantly increased *via* overexpression of DGCR8 in TNBC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

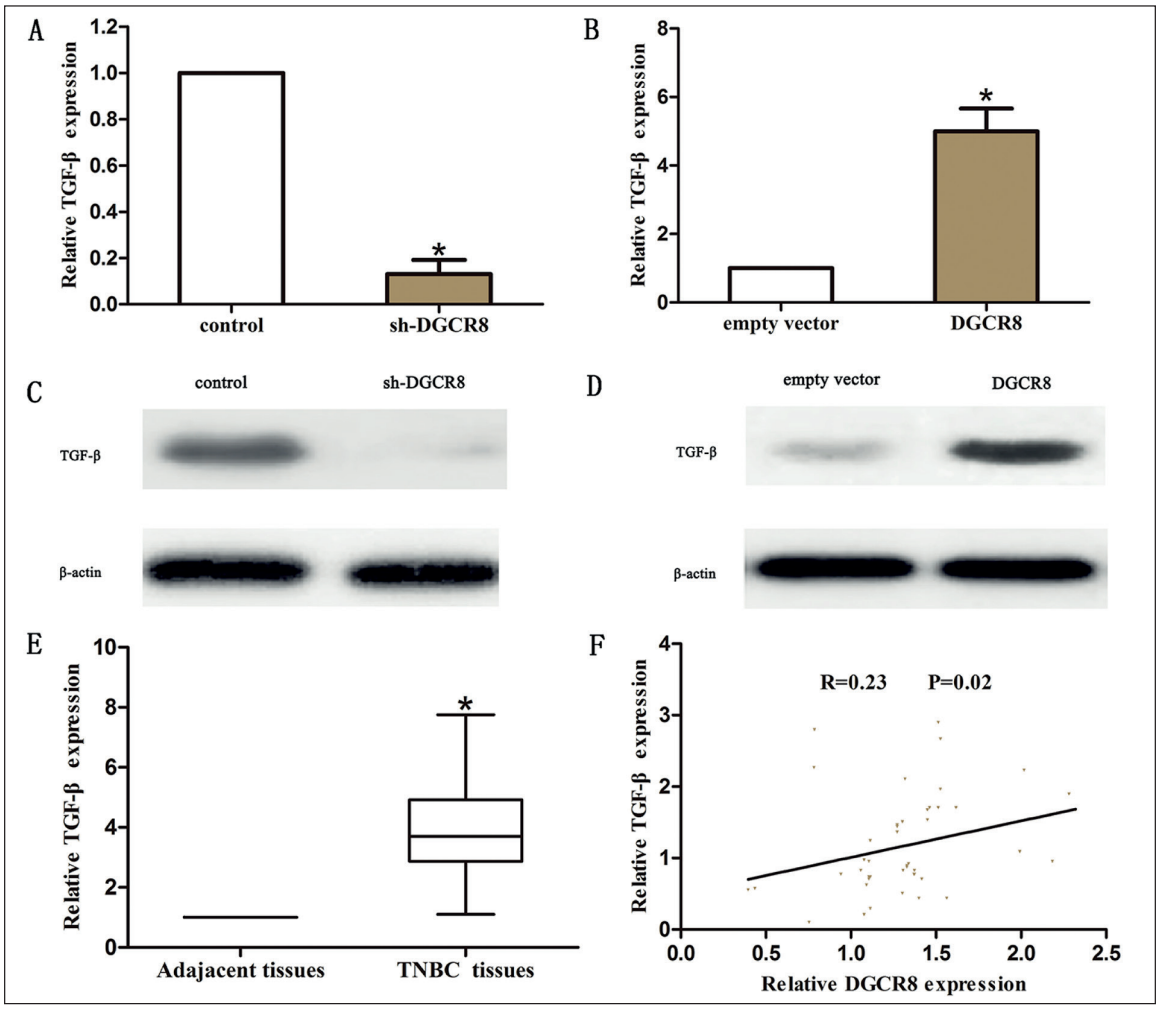

Figure 4. Association between DGCR8 and TGF-β in TNBC cells and tissues. **A,** qRT-PCR results showed that TGF-β expression was lower in DGCR8 shRNA (sh- DGCR8) group compared with the negative control (control) group. **B,** qRT-PCR results showed that TGF-β expression was higher in DGCR8 lentivirus (DGCR8) and the empty vector group. **C,** Western blot assay revealed that TGF-β protein expression was decreased in DGCR8 shRNA (sh-DGCR8) group compared with the negative control (control) group. **D,** Western blot assay revealed that TGF-β protein expression was increased in DGCR8 lentivirus (DGCR8) and the empty vector group. **E,** TGF-β was significantly upregulated in TNBC tissues compared with adjacent tissues. **F,** The linear correlation between the expression level of TGF-β and DGCR8 in TNBC tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05.

sues (Figure 4E). In addition, correlation analysis demonstrated that TGF-β expression level was positively correlated to DGCR8 expression in TNBC tissues (Figure 4F).

Discussion

TNBC is a group of breast cancer characterized by early onset age, strong invasiveness, and high risk of early metastasis, accounting for 10-20% of BC, in which the expressions of ER, PR, and HER2 are lacked. Its biological behavior is highly invasive. Histologically, TNBC is usually a high-grade tumor with high recurrence risk and mortality[9]. Chemotherapy is the only standard treatment at present, but the curative effect lasts for a short time. Therefore, exploring targeted therapy and combination therapy of TNBC has always been a new challenge.

Distant metastasis in early phases of development is a typical biological characteristic of cancer cells. Recent studies have confirmed that abnormal expression of DGCR8 can cause changes in the expression of microRNAs, thus participating in the occurrence and development of

tumors and affecting the prognosis of tumors. In our study, we first confirmed that DGCR8 is abnormally expressed in TNBC specimens. Besides, the knockdown of DGCR8 inhibited cell migration and invasion in TNBC cells, while the overexpression of DGCR8 promoted cell migration and invasion in TNBC cells. The above results indicated that DGCR8 promotes the metastasis of TNBC and may act as an oncogene.

To further identify the underlying mechanism of DGCR8 affecting TNBC cell proliferation and invasion, we predicted and picked TGF-β as the potential target protein of DGCR8 by using bioinformatic analysis and experimental verification. Transforming growth factor-β1 (TGF-β1) is a cytokine with many functions, which participates in many biological processes. It has been reported that TGF-β1 plays an important role in promoting proliferation and metastasis in many types of tumors, including thyroid cancer[10], breast cancer[11], lung cancer[12]. By bioinformatics analysis of the role of microRNAs and their downstream target genes, researchers drew a network diagram of TGF-β1 regulating the proliferation and metastasis of renal cell carcinoma through microRNAs, suggesting that TGF-β1 has a powerful role in promoting tumor progression and may be a potential target for treatment[13].

In the present study, it was found that TGF-β expression could be downregulated after the knockdown of DGCR8, while it could be upregulated after the overexpression of DGCR8. Moreover, TGF-β had significantly high expression in TNBC tissues compared with that of adjacent tissues. TGF-β expression level was positively correlated to DGCR8 expression in TNBC tissues. All the results above suggested that DGCR8 may promote metastasis of TNBC *via* upregulating TGF-β.

Conclusions

Above data identified that DGCR8 is remarkably upregulated in TNBC patients. Besides, DGCR8 can promote the migration and invasion of TNBC cells by upregulating TGF-β. These findings suggest that DGCR8 may serve as a candidate therapeutic target for TNBC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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