

CD151 promotes breast cancer metastasis by activating TGF- β 1/Smad signaling pathway

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Abstract. – **OBJECTIVE:** This study aimed to explore the expression characteristics of CD151 in breast cancer (BC) and to further study its role in the development of BC and potential regulatory mechanisms.

PATIENTS AND METHODS: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to detect the level of CD151 in 82 pairs of BC tissues and adjacent normal ones, and the relationship between CD151 expression and BC pathological parameters and prognosis was analyzed. CD151 expression in BC cells was further validated using qRT-PCR. The CD151 knockdown model was constructed in BC cell lines including MCF-7 and SKBR3 using small interference RNA. The cell counting (CCK-8) and transwell assay were used to analyze the effect of CD151 on the biological function of BC cells, and finally Western blot was performed to explore its underlying mechanism.

RESULTS: QRT-PCR analysis revealed that CD151 level in BC tissues was significantly higher than that in normal ones, and the difference was statistically significant. Compared with patients with low CD151 level, patients with high CD151 level had worse tumor stage, lymph node metastasis, and distant metastases. The high incidence of metastasis lowered the overall survival rate. Compared with the negative control group, the ability of cell proliferation and migration in the CD151 knockdown group was significantly reduced. In addition, Western blot results demonstrated that the levels of proteins in TGF- β 1/Smad pathway, including transforming growth factor- β 1 (TGF- β 1), p-Smad2, p-Smad3, N-cadherin, and MMP-9, were remarkably decreased in cells of si-CD151 group.

CONCLUSIONS: Expression of CD151 in BC was significantly increased, which was found to be associated with BC stage, lymph node or distant metastasis, and poor prognosis. CD151 may promote the proliferation and invasion of BC by regulating TGF- β 1/Smad pathway.

Key Words.

CD151, TGF- β 1/Smad signaling pathway, Breast cancer, Prognosis, Metastasis.

Introduction

Breast cancer (BC) is a common malignancy that seriously threatens human life and health¹⁻³. According to published data, about 15% of new female malignancy cases in China are breast cancer, which has become the highest incidence of female malignant tumors in China, making it the leading cause of death for women under 45-year-old^{4,5}. More recently, the research data show that the morbidity and mortality of breast cancer in China are rising, which has seriously jeopardized women's health and even life⁴⁻⁶. The morbidity and mortality of patients with solid tumors are generally caused by the loss of the normal functions of the disseminated tumor cells. The migration of tumor cells has become a hot topic in the study of the mechanism of cancer metastasis⁷⁻¹⁰. Compared with primary tumors, metastatic tumors cannot be surgically removed and are resistant to chemotherapy, and 90% of cancer deaths are caused by distant metastases^{11,12}. However, the molecular mechanism mediating the migration of breast cancer cells remain elusive, and the molecular markers that predict their metastasis are still limited^{8,10}. The discovery of the key molecules and regulatory pathways in the process of breast cancer progression and metastasis is the focus of breast cancer research, and it can help to guide the early diagnosis and treatment of breast cancer to reduce the mortality and improve the prognosis^{8,10}. Because the pathogenesis has not yet been fully elucidated, the difficulty in diagnosis and treatment is one of the important reasons for its high morbidity and mortality. Therefore, it is of great importance to elucidate the molecular mechanism of breast cancer metastasis and to analyze the prediction, diagnosis, and prognosis of breast cancer metastasis.

CD151 is a member of the trans-membrane protein superfamily (TM4SF). By binding spe-

cifically to integrins, CD151 is involved in many pathophysiological processes such as cell adhesion, migration, and proliferation. It has been demonstrated¹³⁻¹⁶ that CD151 is overexpressed in various tumors and can participate in tumor invasion and metastasis, and its expression level is closely related to the pathological grade, clinical stage, and prognosis of some tumors. But no reports have been reported its expression in BC. Transforming growth factor- β 1 (TGF- β 1) induces hypertrophy of mammary gland and promotes the accumulation of extracellular matrix (ECM) through autocrine and paracrine pathways^{17,18}. Recently discovered Smad protein is the only known intracellular TGF- β 1 receptor substrate that mediates the intracellular signal transduction process of TGF- β 1^{19,20}. Therefore, in this study, the molecular mechanism of whether CD151 mediates the invasion and metastasis of BC via TGF- β 1/Smad signaling pathway is explored to provide experimental basis for its clinical application.

In this study, we detected the expression of lncRNA CD151 in 82 pairs of BC tissues and adjacent tissues, and measured the levels of CD151 protein and mRNA in breast cancer and normal tissues. Additionally, the clinicopathological factors were estimated to explore the effect of CD151 on the biological function of tumor cells through TGF- β 1/Smad signaling pathway.

Patients and Methods

Patients and BC Samples

We collected tumor and paracancerous specimens from 82 pairs of surgically resected breast cancer patients. According to the 7th edition of UICC/AJCC breast cancer TNM staging criteria, all included patients were diagnosed with BC by postoperative pathological analysis and did not preoperatively accept anti-tumor treatment such as radiotherapy or chemotherapy. Ethical Committee of Second Affiliated Hospital of Dalian Medical University approved this research. All the patients have been fully informed of the purpose of the study and signed relevant informed consent forms.

Cell Lines and Reagents

The human breast cancer cell lines (MCF-7, MDA-MB-231, and SKBR3) and normal mammary epithelial cell line, MCF-10A, were purchased

from ATCC (American Type Culture Collection, Manassas, VA, USA). High-glucose Dulbecco's Modified Eagle Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from the Life Technologies (Gaithersburg, MD, USA). The cells were cultured with DMEM containing 10% FBS (Gibco, Rockville, MD, USA) in 37°C, 5% CO₂ incubator.

Transfection

Negative control (si-NC) and siRNA containing the CD151 sequence (si-CD151) were purchased from Shanghai Zima (Shanghai, China). Cells were seeded in 6-well plates, and when they reached 70% of confluence, siRNA transfection was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected after 48 hours for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assays

The cells after 48 h of transfection were harvested and the cells were seeded into 96-well plates at 1000 cells/well. The cells were cultured for 6 h, 24 h, 48 h, and 72 h, and then added with cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan). After incubation for 2 hours, the optical density (OD) value of each well at 490 nm absorbance wavelength was measured by a microplate reader.

Transwell Assay

After 48 hours of transfection, the cells were trypsinized and resuspended in serum-free medium. After cell counting, the diluted cell density was adjusted to 2.0×10^5 /mL. Transwell chambers containing Matrigel reagent were placed in 24-well plates. 200 μ L of the cell suspension was added to the upper chamber, and 500 μ L of medium containing 10% FBS was added to the lower chamber. After 48 hours, the chamber was taken out, and 4% paraformaldehyde was used to fix it for 30 minutes. Crystal violet was used to stain cells for 15 minutes and then washed with phosphate-buffered saline (PBS). The inner surface of the basement membrane of the chamber was carefully cleaned to remove inner cells. Microscopically stained transmembrane cells were visualized in the basement membrane of the chamber and 5 fields were randomly selected to count.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from BC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) using Primescript RT Reagent. The qRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System. The following primers were used for qRT-PCR reactions: CD151: forward: 5'-CTCACAGGACTGGCGAGAC-3', reverse: 5'-ACAGCCCCAATGACCCTCA-3'; β -actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA).

Western Blot

The transfected cells were lysed using a cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 4°C, 14,000 g for 15 minutes. The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies were TGF- α , p-Smad2, p-Smad3, N-cad, Vimentin, MMP-9, and β -actin. The secondary antibodies were anti-mouse and anti-rabbit. Both were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used to process the data, which were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Continuity variables were measured using the t -test and categorical variables were analyzed using the χ^2 -test or the Fisher's exact probability method. Kaplan-Meier method was used to evaluate the survival time of patients, and log-rank test was used to compare differences between different curves. $p < 0.05$ was considered to be statistically significant.

Results

CD151 Is Highly Expressed in BC Tissues and Cell Lines

We detected the level of CD151 in 82 pairs of BC tissues and their adjacent tumor-free tissues

and BC cell lines by qRT-PCR method. The results showed that the expression level of CD151 in BC tissues was significantly higher than that in tumor-free tissues (Figure 1A). Compared with the normal mammary epithelial cells, CD151 was significantly higher in BC cells, and the difference was statistically significant (Figure 2C). The CD151 level was the highest respectively in MCF-7 and SKBR3 cells, so we chose these two cells for subsequent experiments.

CD151 Expression Was Correlated With Clinical Stage, Lymph Node and Distance Metastasis and Overall Survival in BC Patients

Based on qRT-PCR results of 82 pairs of CD151 expression in BC tissues and paracancerous tissues, we divided CD151 expression into high expression group and low expression group, and counted the number of each group. Chi-square test was used to analyze the relationship between CD151 and basic indicators of BC patients. As shown in Table 1, high expression of CD151 was significantly correlated with BC clinical stage, lymph node metastasis, and distant metastasis, but not with age, gender, and tumor location. In order to figure out the interaction between CD151 and the prognosis of patients with RCC, we collected relevant follow-up data. Kaplan-Meier survival curves showed that high CD151 expression was remarkably associated with poor BC prognosis. The higher the CD151 expression, the worse the prognosis was ($p < 0.05$, Figure 1D). This result suggested that CD151 might be a new biological indicator for predicting BC prognosis.

Inhibition of CD151 Inhibited Cell Proliferation

To explore the effect of CD151 on the proliferative capacity of BC cells, we first successfully constructed a CD151 interference expression model (Figure 2B), and used CCK8 to detect their proliferation in si-NC and si-CD151 group. As shown in Figures 2C and 2D, the cell proliferation rate of si-CD151 group was significantly decreased as compared with si-NC group.

Knockdown of CD151 Inhibited Cell Migration and Invasion

We used transwell experiments to explore the effects of CD151 on BC cell migration and invasion. The results of the migration experiment showed that compared with the si-NC group, the

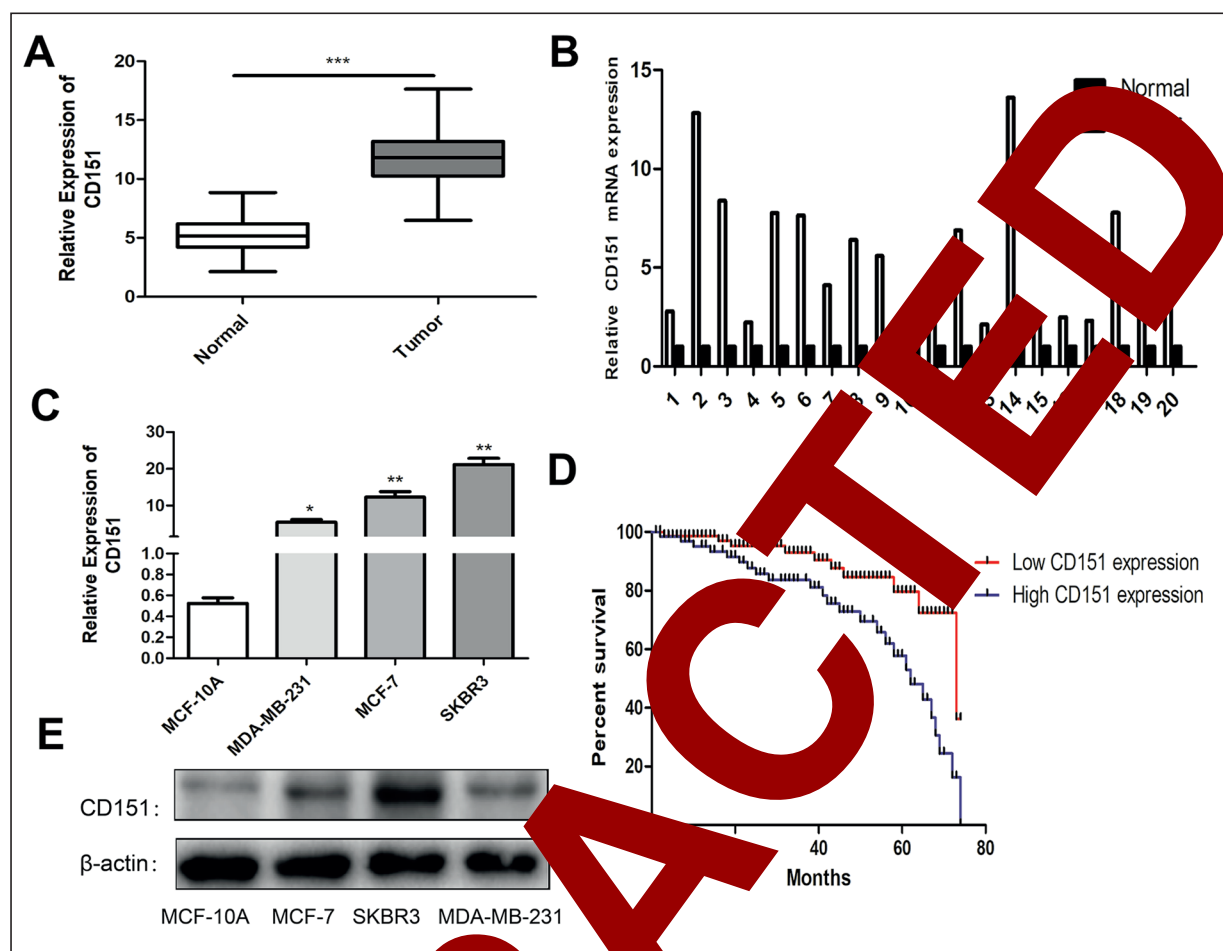


Figure 1. *A, B*, The expression of CD151 in 82 pairs of ESCC tissue was significantly decreased by qRT-PCR. *C*, Expression levels of CD151 in 3 BC cell lines (MCF-10A, MDA-MB-231 and SKBR3) and normal mammary epithelial cell line (MCF-10A) by Western blot and qRT-PCR. *D*, Kaplan-Meier survival curves of patients with BC based on CD151 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in high expression group. A representative data set is displayed as mean $\pm$ SD values. $^{\#}p < 0.05$, $^{**}p < 0.01$.

amount of BC cells transferring the membrane in the transwell chamber was significantly reduced after knocking down CD151, suggesting that the migratory ability was inhibited. The results of the invasion experiment were consistent with this (Figure 2).

Knockdown of CD151 Inhibited the Expression of TGF- β 1/Smad Signaling Pathway

To analyze the potential mechanism of CD151 in promoting cell proliferation and invasion or migration, we detected the changes in expression of the key proteins in the TGF- β 1/Smad pathway by Western blot, including TGF- β 1, p-Smad2,

p-Smad3, N-cad, Vimentin, and MMP-9. The results showed that CD151 knockdown remarkably reduced the levels of the above proteins (Figure 4).

Discussion

With the increase of cancer incidence and mortality, cancer has become the main cause of death in China²¹. According to authoritative statistics, there were approximately 4292,000 new cases of cancer and 2814,000 cases of cancer deaths in China in 2015, including cancer of lung, stomach, liver, and breast²². Breast cancer accounts for 15%

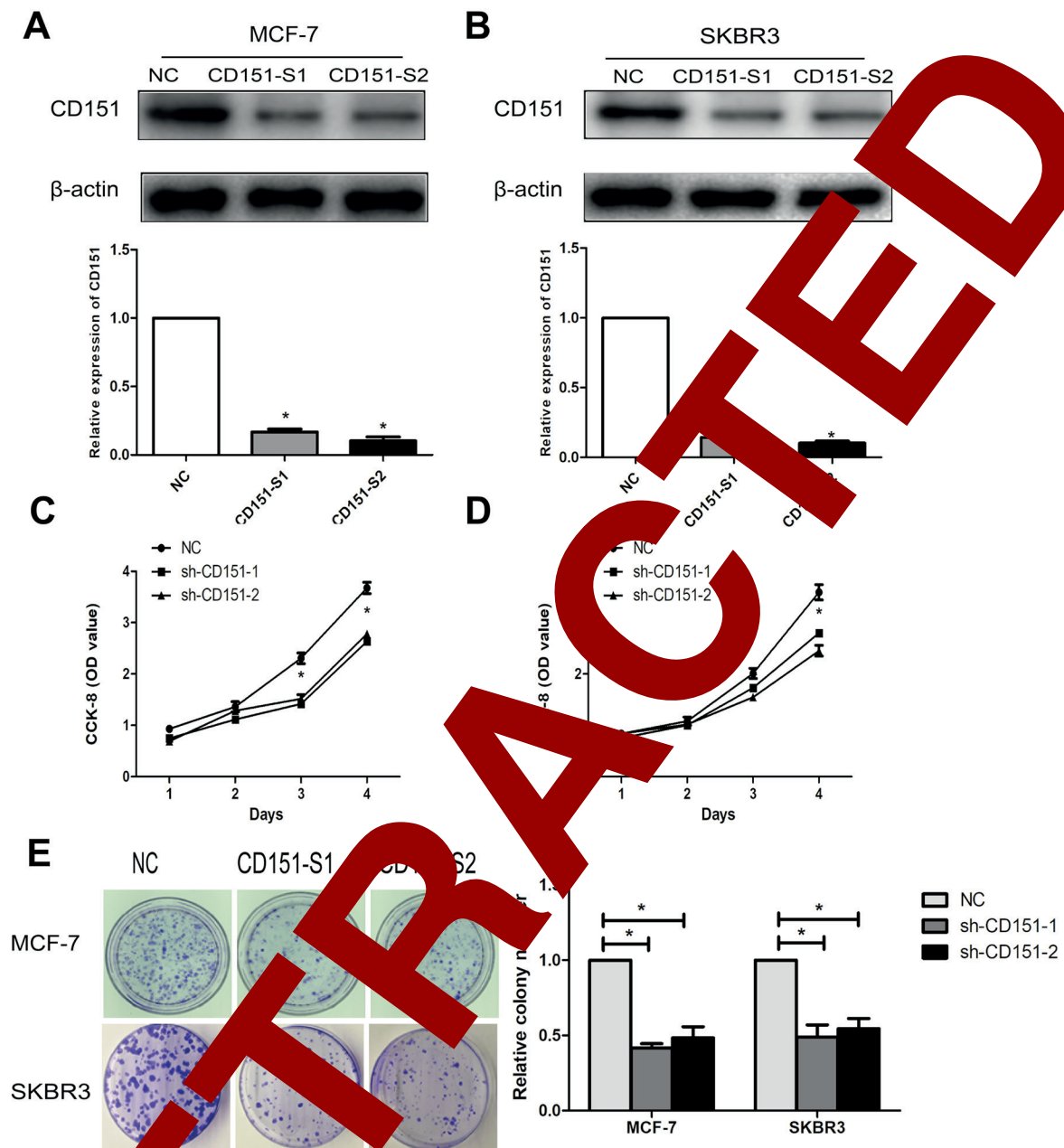


Figure 2. Western blot and qPCR were used to verify the efficiency of CD151 knockdown in MCF-7 and SKBR3 cell lines. **C, D,** Cell growth curve analysis showing the cell growth of MCF-7 and SKBR3 cells with CD151 knockdown. **E, F,** The efficiencies of cell colony formation in MCF-7 and SKBR3 cells with CD151 knockdown. A representative data set is displayed as mean values. * $p < 0.01$.

of all cancers in women, which has become the leading cause of death in women under 45²². BC is one of the common malignant tumors in the world. In recent years, the incidence and mortality of BC in China have gradually increased, and

the early diagnosis rate of BC patients is extremely low. Most of them have developed into middle or late stage when they were diagnosed with BC^{22,23}. The research on early diagnosis, metastasis, recurrence of BC, and adjuvant treatment

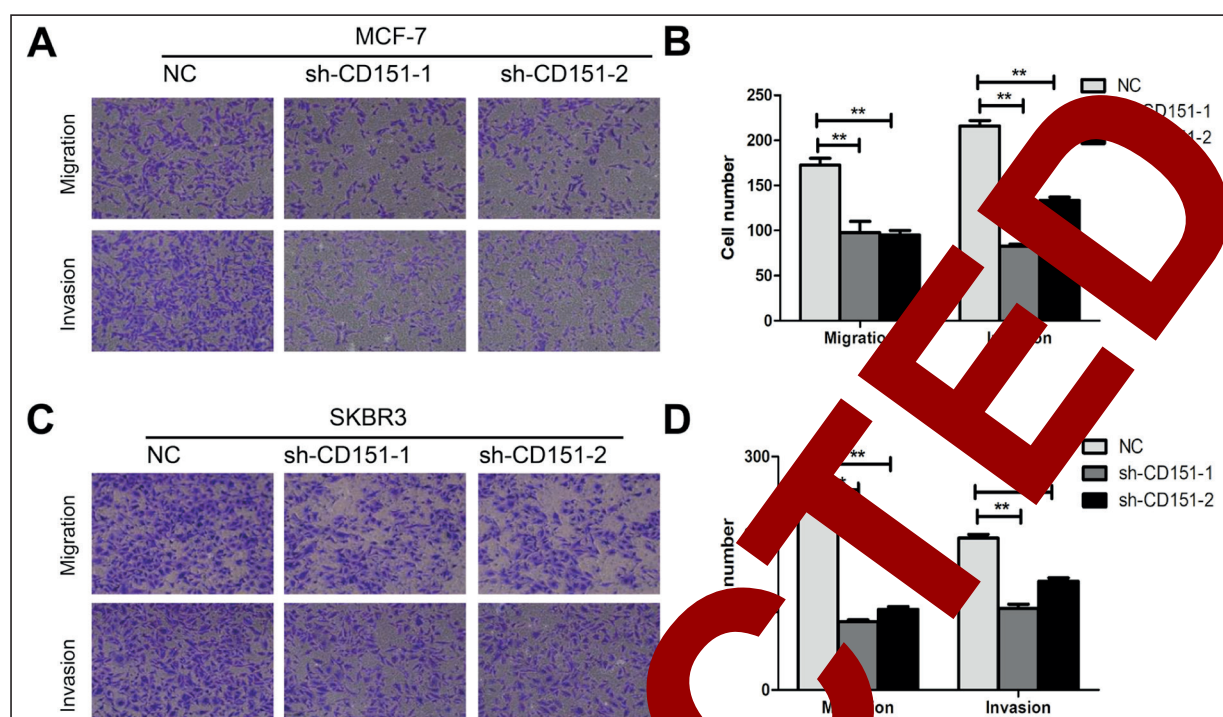


Figure 3. *A, B*, MCF-7 cells transfected with CD151 displayed significantly lower migration and invasion capacity. *C, D*, SKBR3 cells transfected with CD151 displayed significantly lower migration and invasion capacity. A representative data set is displayed as mean $\pm$ SD values. * $p < 0.05$, ** $p < 0.01$.

on advanced BC have become the focus of current research²³. CD151 has been observed to be essential in different diseases, like cancer, but it

unclear whether CD151 exert vital effect on the diagnosis, treatment, and prognosis of BC^{14,24,25}. Therefore, studying the role of CD151 in BC and analyzing its correlation with clinical prognosis will help to improve the diagnosis and treatment of BC as well as the clinical prognosis of patients.

The CD151 gene is the earliest reported oncogene in the TM4SF family and is located on human chromosome 11P15.5. In addition to being widely expressed in normal tissues, the protein product of CD15 gene can be present in lots of tumor tissues, such as cancer of ovary, breast, pancreas and rectum^{13-16,24,25}. Several studies^{13-16,24,25} have showed that the differential expression of CD151 in tumor tissues is closely related to tumor metastasis and prognosis. Studies have found that the expression of CD151 in prostate cancer is evidently higher than that in benign prostatic hyperplasia using immunohistochemical assay, and CD151 staining is strong in poorly differentiated prostate cancer tissues, while in well-differentiated prostate cancer tissues, its staining is weak. High level of CD151 indicates poor prognosis^{26,27}. In addition, the use of immunohistochemistry and RT-PCR in the detection of CD151 in patients with colon cancer patients



Figure 4. Knockdown of CD151 expression significantly decreased the expression of TGF- β 1/Smad signal pathway, including TGF- β 1, p-Smad2, p-Smad3, N-cad, Vimentin, and MMP-9.

combined with following-up the survival time of patients has demonstrated that CD151 gene and protein expression was significantly higher in 81 (81/146) cases, and patients with low expression of CD151 had significantly higher 3-year survival than those with high CD151 expression^{28,29}. We explored the expression of CD151 in BC and its role in the development of BC. We verified CD151 level in 82 pairs of BC tissues and adjacent ones. It was found that CD151 was markedly up-regulated and positively correlated with BC stage, lymph node metastasis, distant metastasis, and poor prognosis. Therefore, we suggested that CD151 might have an influence on promoting the occurrence of BC. To further explore the effect of CD151 on the biological function of BC, we constructed a CD151 knockdown expression model using small interfering RNA. The results of CCK8, invasion and migration experiments showed that CD151 could indeed accelerate the progression of BC. Nevertheless, its specific mechanism remains elusive.

The TGF- β /Smad signaling pathway has been found in human and various biological tissues. Its main function includes transduction of complex receptor signals on the cell surface through endocrine and paracrine pathways, regulating cell growth, differentiation, apoptosis, adhesive, and other functions³⁰⁻³². Scholars^{17,18} have found that TGF- β superfamily growth differentiation factors play an important role in embryonic development, wound repair, immune reaction, inflammatory response, fibrosis, bone formation, and reconstruction. Smad is a family of proteins that share homology with the Drosophila MAD and Smad in nematodes and can interact with other DNA binding proteins to regulate the transcription of target proteins³⁰. There are many cytokines involved in the formation of BC, such as angiotensin II, endothelin-1, TGF- β , etc.³³. The TGF- β /Smad signaling pathway is a classical signaling pathway that functions not only to affect cell proliferation and differentiation, but also to embryonic development, bone formation and remodeling, and extracellular matrix formation³⁴. The TGF- β /Smad signaling pathway mainly includes a three-part structure, namely extracellular matrix protein, TGF- β receptor protein (T3R) on the cell membrane, and intracellular Smad protein. According to the order of signal transduction, extracellular TGF- β binds to the extracellular domain of T3R on the cell membrane, activates the serine/threonine kinase, and activates type I receptors that interact with

intracellular substrates and transduce signals into cells. The receptor substrate in the cell is mainly the Smad protein family, and Smad plays a specific role. There are 9 Smad proteins in the Smad protein family in cells, and they are divided into 3 categories according to their roles in BC. The first category is called receptor-regulated Smads (R-Smads), which mainly include Smad1, Smad2, Smad3, Smad5, Smad8 and Smad9. Their main function is to promote TGF- β ^{19,20}. The second class is called the common pathway type Smad (Co-Smad), and there is only one member Smad4, in this class, which is a common molecule required for TGF- β signaling process. The third type is inhibitory Smad (I-Smad), mainly including Smad6 and Smad7, whose main function is to inhibit the regulation of TGF- β family protein signal transduction, to exert a regulatory effect on the development of BC²⁰.

To explore whether CD151 could promote the development of BC by regulating TGF- β /Smad, we analyzed the levels of key proteins including TGF- β 1, p-Smad2, p-Smad3, N-cad, Vimentin, and EMT in TGF- β /Smad pathway after knocking down CD151. Results indicated these proteins were evidently reduced after knocking down CD151, suggesting a positive regulatory relationship between CD151 and TGF- β /Smad pathway.

Conclusions

We showed that the expression of CD151 in BC was significantly increased, which was evidently associated with BC stage, lymph node metastasis, distant metastasis, and poor prognosis. CD151 may promote the proliferation and invasion of BC by regulating TGF- β /Smad pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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