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Long non-coding RNA TTN-AS1 promotes the metastasis in breast cancer by epigenetically activating DGCR8

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Abstract. – OBJECTIVE: Breast cancer (BC) is one of the most common fatal cancers. Recent studies have identified the vital roles of long non-coding RNAs (lncRNAs) in the development and progression of BC. This research aimed to investigate the underlying mechanisms of lncRNA TTN-AS1 in the metastasis of BC.

PATIENTS AND METHODS: TTN-AS1 expression of tissues was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) in 50 BC patients. Wound healing assay and transwell assay were used to observe the metastatic alteration of BC cells after knockdown or overexpression of TTN-AS1. Moreover, RT-qPCR and Western blot assay were performed to discover the potential targets of TTN-AS1 in BC.

RESULTS: TTN-AS1 expression in BC samples was significantly higher than that in adjacent tissues. Besides, the migration and invasion of BC cells were markedly inhibited after TTN-AS1 was silenced, while promoted after TTN-AS1 overexpression. In addition, the decrease of DGCR8 was observed after TTN-AS1 was inhibited in BC cells, while DGCR8 was upregulated after overexpression of TTN-AS1. Furthermore, DGCR8 expression showed a significant enhancement in BC tissues and was positively associated with TTN-AS1 level.

CONCLUSIONS: Our study discovered a new oncogene in BC and suggested that TTN-AS1 could enhance BC cell migration and invasion *via* sponging DGCR8, which provided a novel therapeutic target for the treatment of breast cancer.

Words: long non-coding RNA, TTN-AS1, BC, DGCR8.

Introduction

Breast cancer (BC) is one of the most frequent malignancy diagnosed in women and remains the second most common cause of cancer-related death in women globally[1]. Approximately 246,660 new cases of breast cancer were diagnosed and 40,450 cases succumbed to breast cancer in the USA in 2016[2]. The prognosis for patients with breast cancer is strongly related to the stage of the disease at diagnosis. Although great improvements have been made in the treatment of BC for the past decades, the outcome of patients with BC remains poor with the 5-year survival rate below 25%[3]. Therefore, it is important to have a deeper understanding of the molecular mechanism underlying BC and improve the poor prognosis of the patients.

Long non-coding RNAs (lncRNAs) are a class of transcripts with more than 200 nucleotides in length. LncRNAs have been reported to play a crucial role in carcinogenesis and gene regulation. For example, lncRNA TP73AS1 promoted cell apoptosis and inhibited cell proliferation in colorectal cancer by serving as a competing endogenous RNA for miR-103[4]. LncRNA FALEC also facilitated cell proliferation in melanoma by silencing p21 and was closely associated with poor prognosis for patients with melanoma[5]. By targeting miR-221/SOCS3, lncRNA GAS5 suppressed cell proliferation, cell metastasis and gemcitabine resistance in pancreatic cancer[6]. In addition, lncRNA TUG1 could enhance cell proliferation and inhibit cell apoptosis in human osteosarcoma by downregulating SOX4[7].

However, the clinical role and underlying mechanisms of TTN-AS1 in the development of BC remain unexplored. In the present study, we explored whether TTN-AS1 functioned in the metastasis of BC.

Patients and Methods

Patients and Clinical Samples

A total of 50 BC patients who received surgery at the Xingtai People's Hospital were enrolled in

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this study. Before the operation, informed consent was achieved. None of the patients received radiotherapy or chemotherapy before the operation. Tissues harvested from the surgery were stored immediately at -80°C. This study was approved by the Ethics Committee of Xingtai People's Hospital. Informed consents were obtained from all participants before the study.

Cell Culture

Human BC cell lines (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 culture medium consisted of 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA), as well as 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, 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; Bio[illegible] Inc., San Diego, CA, USA) against TTN-A[illegible] Negative control shRNA were synthesize[illegible]d transfected into LCC9 BC cells. Besides, len[illegible] rus (Biosettia Inc., San Diego, CA, USA) agai[illegible] TTN-AS1 (TTN-AS1) was synt[illegible]d to exog[illegible] enously upregulated in SKB[illegible]s. After 48 h transfection, Real Ti[illegible]quanti[illegible]e Polymerase Chain Reaction [illegible]PCR) [illegible]d to measure the transfecti[illegible] ef[illegible]

RNA Extraction [illegible] Real Tim[illegible]ntitative Polymerase C[illegible]action

Total RNA[illegible]s ext[illegible]d from the tissues and cells using[illegible] TRIzol k[illegible]vitrogen, Carlsbad, CA, US[illegible]espectively, follo[illegible]by measurement of RN[illegible]ncentr[illegible]ion using an [illegible]traviolet spectrophot[illegible]ter (H[illegible]n, Tokyo, Japan). The complemen[illegible]De[illegible]bose [illegible]leic Acid (cDNA) was synthes[illegible]cordin[illegible]e instructions of the Pri-[illegible]ipt[illegible] M[illegible] Mix kit (Invitrogen, Carlsbad, CA, USA). [illegible]mocycling conditions were as foll[illegible] pre-dena[illegible]uration at 95°C for 5 min, denatu[illegible]C for 10 s, annealing at 60°C for 30 [illegible]r a total of 35 cycles. Following are the primers [illegible]ng for RT-qPCR: TTN-AS1, forward 5′-TCCT-[illegible]CATCACCTAGCC-3′ and reverse 5′-GAT-GC[illegible]GAAGTAGAGTCATTGG-3′; β-actin, forward 5'-CCAACCGCGAGAAGATGA-3' and reverse 5'-CCAGAGGCGTACAGGGATAG-3'.

Wound Healing Assay

Totally 1.0 × 10^4 cells were seed into a 6-well plate. Three parallel lines were made on the back of each well. After growing to a[illegible] of confluent, cells were scratched w[illegible] pipet[illegible] tip. The cells were photographed[illegible]der a light microscope (Leica, Wetzlar, Ger[illegible]) h and 48 h after scratching. Each assay was [illegible]dently repeated in triplicate.

Transwell Assay

After 24 hours' tra[illegible]tion [illegible]10^5 cell[illegible]in 100 µL of serum-free[illegible]M[illegible] transf[illegible]ed to the top chambe[illegible] µm cu[illegible]inser[illegible]orning, Lowell, NY[illegible]) coated wi[illegible] of Matrigel (BD Bi[illegible] Franklin L[illegible], NJ, USA). Then, 20[illegible]BS-D[illegible]M was added to the lower chamber of the cultu[illegible]erts. After 24 hours' inc[illegible] these inserts[illegible]e treated by methan[illegible]r 30 min and stained by hematoxylin for 20 [illegible]. An inverte[illegible]icroscope (×40) was utilized [illegible]ounting inva[illegible]cells in three random fields.

We[illegible] Bl[illegible]nalysis

Reag[illegible]oimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized [illegible]t the protein from cells. Bicinchoninic [illegible]id ([illegible]A) protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody of rabbit anti-β-actin and rabbit anti-DGCR8 (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody of goat anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA) after rinsing with the Tris-Buffered Saline and Tween solution (TBST; Sigma-Aldrich, St. Louis, MO, USA). Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of the 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 statistical significance was defined as $p<0.05$.

Results

TTN-AS1 Expression Level in BC Tissues and Cells

To determine the biological function of TTN-AS1 in the tumorigenesis of BC, we detected TTN-AS1 expression levels in 50 paired BC specimens by RT-qPCR. The results indicated that TTN-AS1 was significantly upregulated in BC tissue samples compared with adjacent tissues (Figure 1A). TTN-AS1 expression was also detected in four BC cell lines and it showed remarkable enhanced expression in BC cells compared to that of MCF-10A (Figure 1B).

Knockdown of TTN-AS1 Suppressed Cell Migration and Invasion in BC Cells

To further investigate whether TTN-AS1 was involved in the metastasis of BC, we knocked down the TTN-AS1 expression in MCF-7 BC cell *via* shTTN-AS1 to explore the potential function of TTN-AS1 *in vitro* (Figure 2A). Furthermore, cell migration was examined *via* wound healing assay after the knockdown of TTN-AS1 in the MCF-7 cells. The results revealed that cell m tion was considerably reduced after the down of TTN-AS1 (Figure 2B). Transwell y results also indicated that the number of inv cells was remarkably decreased after TTN-A was silenced in BC cells (Figur

Overexpression of TTN l Pro ted Cell Migration and I on in

T-47D BC cell lin wa s or pression of TTN-A nd th sfection was detected by RT-q (Figure 3A) eover, the results of the wound healing assay showed that overexpression of TTN-AS1 significantly promoted the cell migration in BC cells (Figure 3B). Transwell assay results also rev the number of invaded cells was re ably i creased after TTN-AS1 was upre ted in BC cells (Figure 3C).

The Interaction Betwe DGCR8 TTN-AS1 in BC

Starbase v2.0 (http rbase. edu.cn/s base2/rbpLncRNA.p as u o iden fy the targets of TTN-A result owed that DGCR8 w gnifica down ated in the sh-TTN-A roup compa ne control group (Fig Meanwhile, CR8 was up-regulated the T S1 group compared with the empty vector gro ure 4B). The results of W blot showed DGCR8 was mark-e downregulated in the sh-TTN-AS1 group pared with t control group, while upregu- in the TTN S1 group compared with the e vector gr (Figure 4C-D). Furthermore, DG in BC tissues was remarkably higher ed with that of the adjacent tis-ues (Figure 4E). In addition, correlation analysis trated that DGCR8 expression level was y correlated to the TTN-AS1 expression in BC tissues (Figure 4F).

Discussion

LncRNAs have been shown to modulate the progression of BC. For example, lncRNA Z38 might function as a potential biomarker of

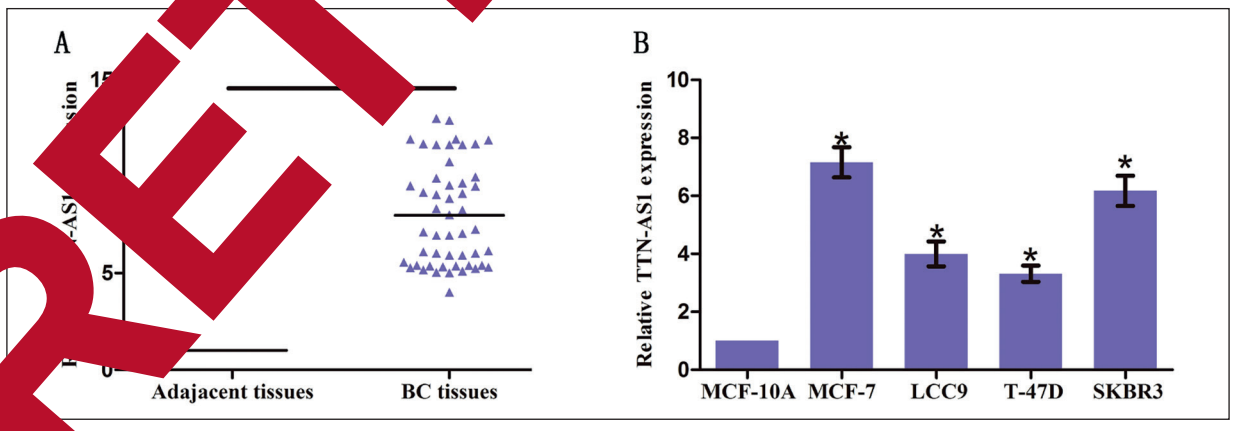

Fig 1. Expression level of TTN-AS1 was increased in BC tissues and cell lines. **A,** TTN-AS1 expression was significantly increased in the BC tissues compared with the adjacent tissues. **B,** Expression levels of TTN-AS1 relative to β-actin were determined in the human BC cell lines and MCF-10A (normal human breast cell line) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

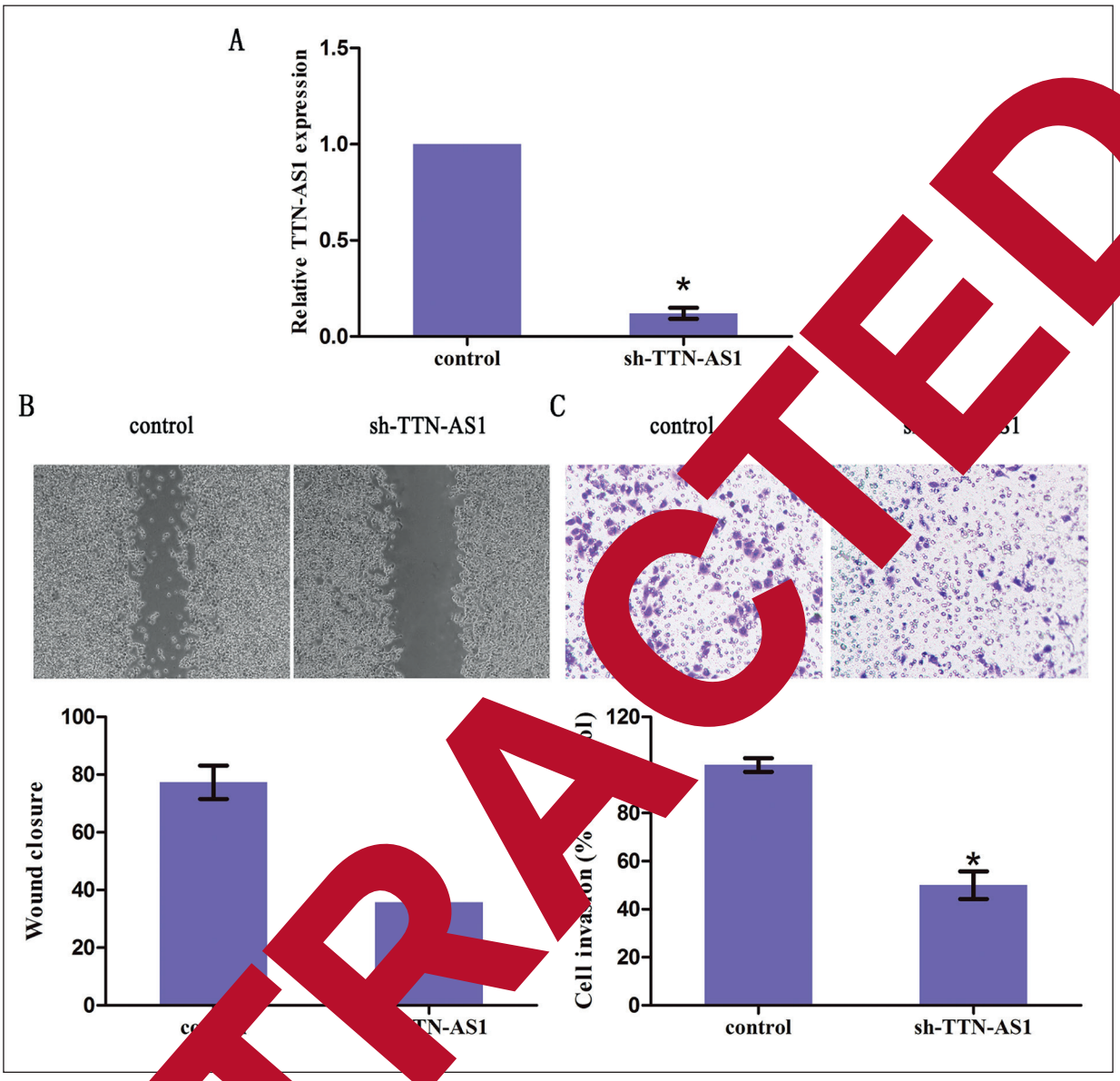

Figure 2. Knockdown of TTN-AS1 inhibited BC cell migration and invasion. **A,** TTN-AS1 expression in BC cells transduced with TTN-AS1 shRNA (sh-TTN-AS1) and the negative control (control) was detected by RT-qPCR. β-actin was used as an internal control. **B,** Wound healing assay showed that silence of TTN-AS1 significantly repressed cell migrated ability of BC cells (magnification: 40×). **C,** The transwell assay showed that the number of invaded cells was significantly decreased *via* silencing TTN-AS1 in BC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error). *$p<0.05$.

BC[illegible]y acting as a sponge to miR-520c-3p, lncRNA HOXA-AS2 promoted cell proliferation and invasion in BC[9]. Besides, lncRNA LINC00511 promoted tumorigenesis and stemness of BC by regulating the miR-185-3p/E2F1/Nanog pathway[10]. LncRNA CASC11 was also reported to contribute to cell proliferation and cell mobility in BC by targeting miR-20b[11].

Distant metastasis in early phases of development is a typical biological characteristic of cancer cells. TTN-AS1 is a novel lncRNA which has been reported to be an oncogene in several tumors, including cervical cancer, esophageal squamous cell carcinoma and papillary thyroid cancer[12-14]. In our study, we first observed that TTN-AS1 was abnormally expressed in BC specimens. Besides, knock-

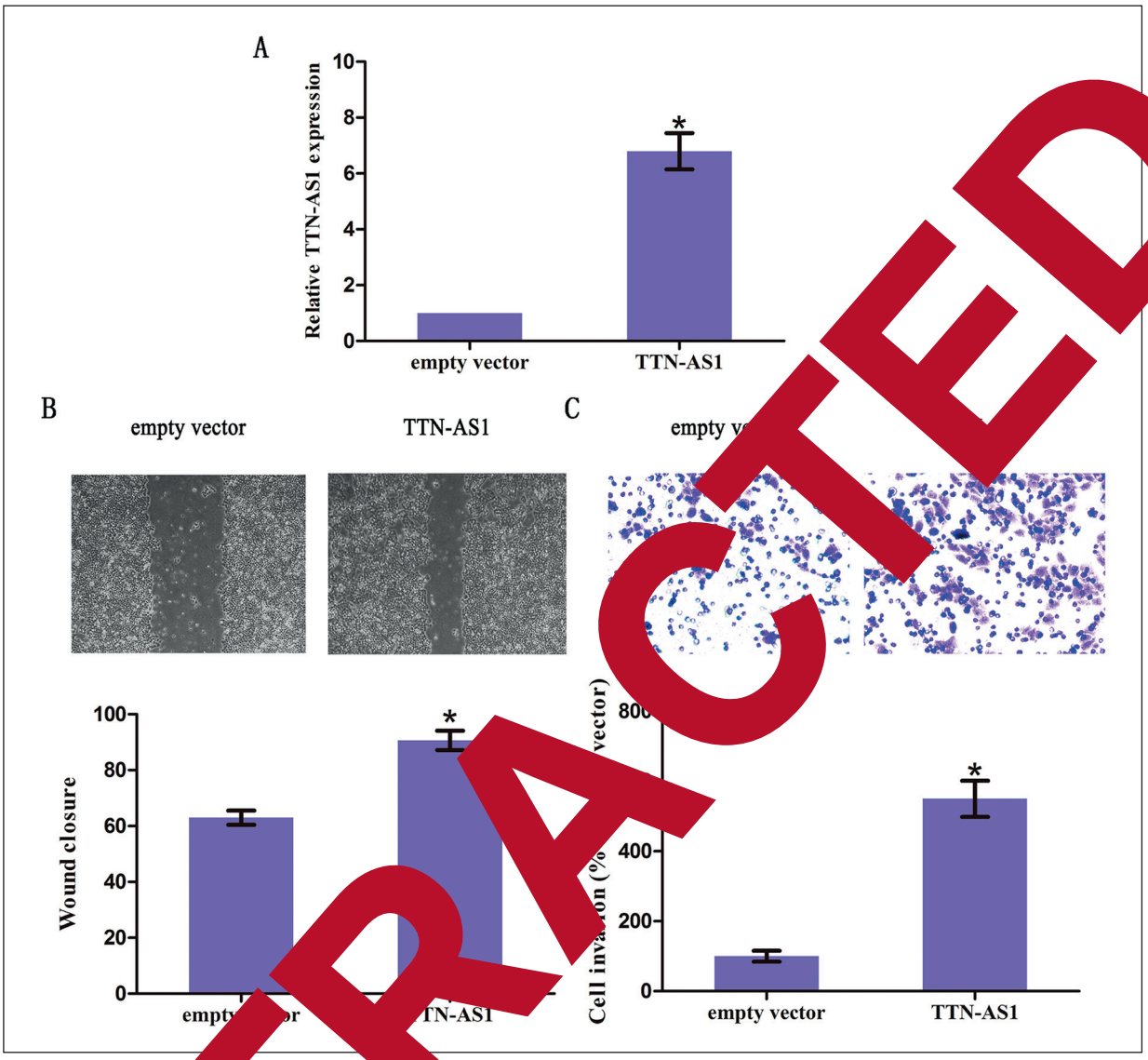

Figure 3. Overe[illegible] of TTN-AS1 pr[illegible]d BC cell proliferation and invasion. **A,** TTN-AS1 expression in BC cells transduced with [illegible]-AS[illegible]irus (TTN-AS1) and the empty vector was detected by RT-qPCR. β-actin was used as an internal cont[illegible]. **B,** Wound [illegible] assay showed that overexpression of TTN-AS1 significantly promoted cell migrated ability of B[illegible]s (magnificatio[illegible] **C,** The transwell assay showed that number of invaded cells was markedly increased *via* over[illegible]ssion of TTN-AS1 i[illegible] cells (magnification: 40×). The results represent the average of three independent experi[illegible]s (mean[illegible]tandard error). *$p<0.05$.

do[illegible]of T[illegible]1 inhibited the migration and [illegible]vasion o[illegible]BC cells, while overexpressi[illegible]AS1 promoted cell migration [illegible]invasion. The above results indicated that [illegible]N-AS1 acted as an oncogene to promote the [illegible]tasis of BC.

[illegible]further identify the underlying mechanism of TTN-AS1 in BC cell proliferation and invasion, we predicted and identified DGCR8 as the potential target of TTN-AS1 by using bioinformatics analysis. RNA binding protein DGCR8 was encoded by the DiGeorge syndrome critical region gene 8 and functions as a critical protein for microRNA (miRNA) biogenesis[15]. Moreover, DGCR8 gene has been reported to participate in the progression of cancers. For example, DGCR8 inhibited the tumor progression of prostate cancer[16]. Besides, knockdown of DGCR8 sup-

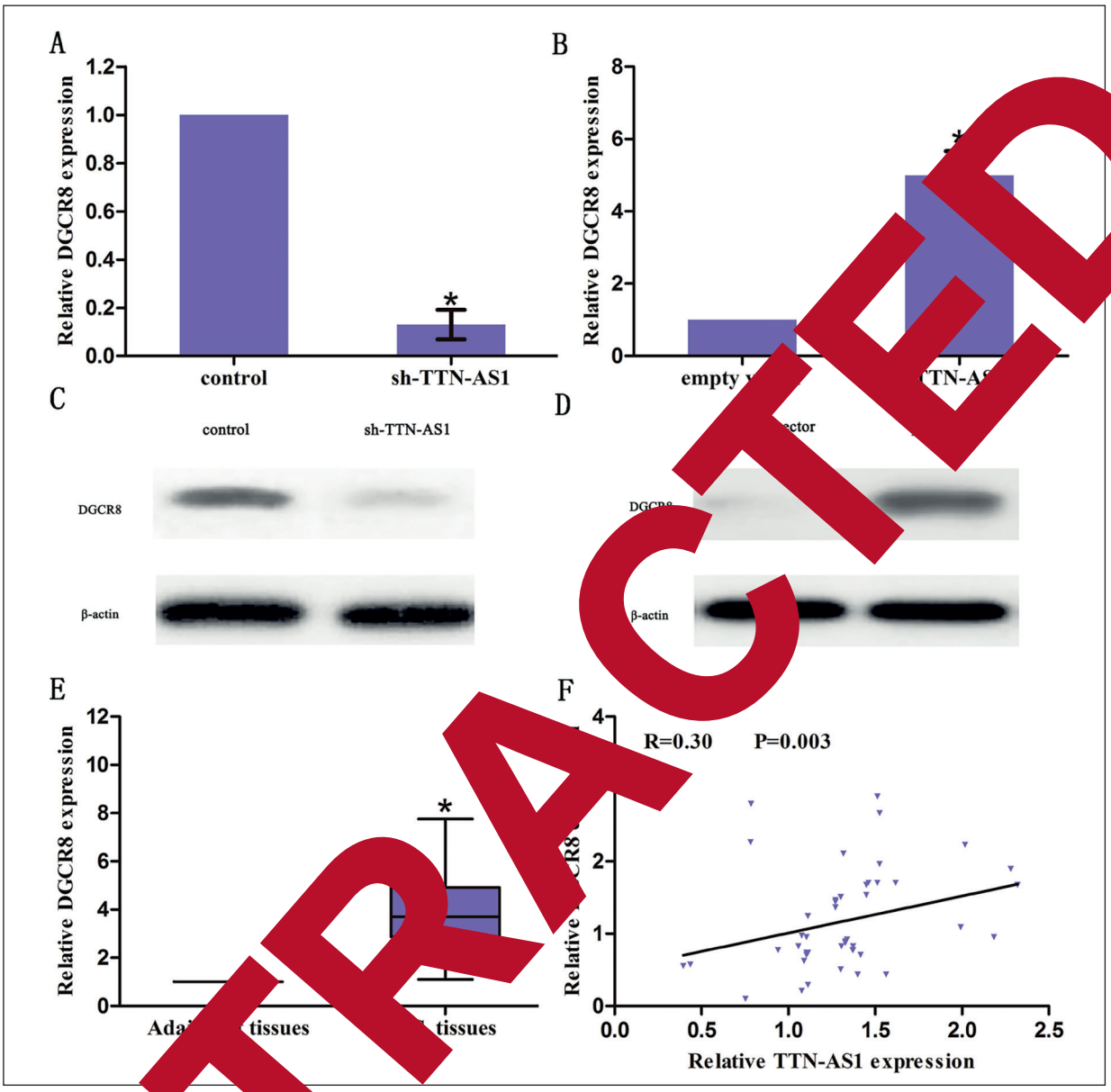

Figure 4. ...ciation betwee... AS1 and DGCR8 in BC cells and tissues. **A,** RT-qPCR results showed that DGCR8 expressio... significantly lower ... TTN-AS1 shRNA (sh- TTN-AS1) group compared with the negative control (control) group. ...-qPCR ...sults showed ...at DGCR8 expression was markedly higher in TTN-AS1 lentivirus (TTN-AS1) and the ... vector ...p. **C,** Western blot assay revealed that DGCR8 protein expression was decreased in the TTN-AS1 shR... TT...1) grou... mpared with the negative control (control) group. **D,** Western blot assay revealed that DGCR8 protei... was inc... in TTN-AS1 lentivirus (TTN-AS1) and the empty vector group. **E,** DGCR8 was significantly ...gulate... tissu... mpared with adjacent tissues. **F,** The linear correlation between the expression level of DGCR8 ...N-AS... ...es. The results represent the average of three independent experiments. Data are presented as the mea... standard... $p<0.05$.

...ssed cell proliferation, cell migration and ...asion in ovarian cancer[17].

...he present work, DGCR8 expression could be ...wnregulated after knockdown of TTN-AS1, while upregulated after overexpression of TTN-AS1. Moreover, DGCR8 exhibited a remarkable increase in BC tissues compared with that of the adjacent tissues. A positive association was also observed between DGCR8 expression and TTN-AS1 expression in BC tissues. All the results above demonstrated that TTN-AS1 might promote metastasis of BC *via* upregulating DGCR8.

Conclusions

In this study, we identified that TTN-AS1 was remarkably increased in BC patients. Besides, TTN-AS1 could promote cell migration and invasion in BC by upregulating DGCR8. These findings suggested that TTN-AS1 might contribute to therapy for BC as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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