Long noncoding RNA TTN-AS1 promotes breast cancer cell migration and invasion via sponging miR-140-5p

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Research

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Abstract

Objectives Breast cancer (BC) is one of the most ordinary fatal cancers. Recent studies have identified the vital role of long noncoding RNAs (lncRNAs) in the development and progression of BC. In this research, lncRNA TTN-AS1 was studied to identify how it functioned in the metastasis of BC.

Methods TTN-AS1 expression of tissues was detected by RT-qPCR in 56 BC patients. Wound healing assay and transwell assay were used to observe the biological behavior changes of BC cells through gain or loss of TTN-AS1. In addition, luciferase assays and RNA immunoprecipitation (RIP) assay were performed to discover the potential targets of TTN-AS1 in BC cells.

Results TTN-AS1 expression level in BC samples was higher than that of adjacent ones. Besides, cell migrated ability and cell invaded ability of BC cells were inhibited after TTN-AS1 was silenced. Cell migrated ability and cell invaded ability of BC cells were promoted after TTN-AS1 was overexpressed. In addition, miR-140-5p was upregulated after silence of TTN-AS1 in BC cells, while miR-140-5p was downregulated after overexpression of TTN-AS1 in BC cells. Furthermore, luciferase assays and RNA immunoprecipitation assay (RIP) showed that miR-140-5p was a direct target of TTN-AS1 in BC.

Conclusion Our study uncovers a new oncogene in BC and suggests that TTN-AS1 could enhance BC cell migration and invasion via sponging miR-140-5p, which provides a novel therapeutic target for BC patients.

Introduction

Breast cancer(BC) is the most frequently malignancy diagnosed and the second-leading cause of cancer-related death in female in the world[1]. It is reported that 246,660 new cases were diagnosed of BC which accounts for 29% of all cancers in women in America in 2016. Moreover, 40,450 cases were estimated to die due to BC in the same year[2]. Despite tremendous advances have been made in the diagnosis and therapeutic management of BC for the last decades, the prognosis for patients with BC remains poor due to the high rate of metastasis[3]. Therefore, it is urgent to have a better understanding of molecular mechanism of pathogenesis in BC and improve the poor prognosis for BC patients.

Most of the genome is transcribed into noncoding RNA (ncRNA) molecules that do not coding proteins. Long noncoding RNAs (lncRNAs) are those transcriptions longer than 200 nucleotides and have been recently reported to exploit multiple modes of action in regulating gene expression and development of cancers. For example, by sponging miR-27b-3p, lncRNA KCNQ1OT1 facilitates cell proliferation and cell invasion in the progression of non-small cell lung cancer via modulating the expression of HSP90AA1[4]. By acting as a sponge to miR-101-3p, lncRNA SPRY4-IT1 promotes the progression of bladder cancer via upregulating the expression of EZH2[5]. LncRNA PVT1 promotes glucose metabolism, cell motility, cell proliferation and tumor progression in osteosarcoma by modulation of miR-497/HK2 axis[6]. LncRNA

MEG8 enhances epigenetic induction of the epithelial-mesenchymal transition in pancreatic cancer cells[7].

However, the clinical role and underlying mechanisms of TTN-AS1 in the development of BC remain unexplored. In the present study, we performed function and mechanism assays to explore whether TTN-AS1 functioned in the metastasis of BC.

Materials And Methods

Patients and clinical samples

56 cases of BC tissues and their adjacent tissues were collected from patients who received surgery at First Affiliated Hospital of Anhui Medical University between 2015-2018. Written informed consent was achieved before surgical resection. No radiotherapy or chemotherapy was performed before the surgery. All tissues were saved immediately at –80°C. An Institutional Review Board of First Affiliated Hospital of Anhui Medical University approved the protocols for using those tissues for our research.

Cell culture

Human BC cell (MCF-7, LCC9, T-47D, SKBR3) and normal human breast cell line (MCF-10A) were got from the American Type Culture Collection (ATCC, USA). Culture medium consisted of 10% fetal bovine serum (FBS; Invitrogen Life Technologies, USA), Dulbecco's Modified Eagle 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 TTN-AS1 was synthesized. Negative control shRNA was also synthesized. TTN-AS1 shRNA (sh-TTN-AS1) and negative control (control) were then used for transfection in LCC9 BC cells. 48h later, RT-qPCR was used to detect transfection efficiency in these cells. Besides, lentivirus (BiosettiaInc., San Diego, CA, USA) against TTN-AS1(TTN-AS1) was synthesized and then used for transfection in SKBR3 BC cells. Empty vector was used as control.48h later, RT-qPCR was used to detect transfection efficiency in these cells.

RNA extraction and RT-qPCR

Total RNA was extracted from cultured BC 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, Japan). Thermocycling conditions was as follows: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, a total of 35 cycles. Following are the primers using for RT-qPCR: TTN-AS1, forwards 5'-TCCTTAGGCATCACCTAGCC-3' and reverse 5'-GATGGAGGAAGTAGAGTCATTGG-3'; β-actin, forward 5'-CCAACCGCGAGAAGATGA-3' and reverse 5'-CCAGAGGCGTACAGGGATAG-3'.

Scratch wound assay

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 about confluent of 90 %, cells were scratched with a pipette tip and cultured in a medium. Cells were photographed under a light microscope after 48 h. Each assay was independently repeated in triplicate.

Transwell assay

8μm pore size insert was provided by Corning (Corning, New York, USA). 4×10^4 cells in 150μL serum-free DMEM were transformed to top chamber of the insert coated with or without 50 μg Matrigel (BD, Bedford, MA, USA). The bottom chamber was filled with DMEM and FBS. 48h later, the top surface of chambers was immersed for 10 min with precooling methanol and was stained in crystal violet for 30 min.

Luciferase assay and RNA immunoprecipitation (RIP) assay

DIANA LncBASE Predicted v.2 was used to predict the potential target microRNAs and fragment sequences containing TTN-AS1 reaction sites. The TTN-AS1 3'-UTR wild-type (WT) sequence named TTN-AS1-WT was 5'-CUUUUCCAUCCUUAAACCACUU-3' and the mutant sequence of TTN-AS1 3'-UTR missing the binding site with miR-140-5p named TTN-AS1-MUT was 5'-CUUUUCCAUCCUUUUUGGUGAU-3'. Luciferase reporter gene assay kits (Promega, Madison, WI, U.S.A.) were used to detect the luciferase activity of BC cells. The luciferase reporter gene vector was constructed, and BC cells were transfected. For RIP assay, Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was performed according to the protocol. Then RT- qPCR was used to detect co-precipitated RNAs. Treated BC cells were collected and lysed using RIP lysis buffer containing protease inhibitor and RNase inhibitor. Cells were incubated with the RIP buffer containing magnetic beads coated with Ago2 antibodies (Millipore). IgG acted as a negative control (input group). After incubation for 2 h at 4°C, co-precipitated RNAs were isolated and measured by RT-qPCR analysis.

Statistical analysis

All statistical analyses were performed by GraphPad Prism 5.0. The difference between two groups were compared by independent-sample t test. The statistically significance was defined as $p<0.05$.

Results

TTN-AS1 expression level in BC tissues and cells

Firstly, TTN-AS1 expression was detected via RT-qPCR in 56 patients' tissues and 4 BC cell lines. As a result, TTN-AS1 was significantly upregulated in BC tissue samples (**Figure 1A**). TTN-AS1 expression level in BC cells was higher than that of MCF-10A (**Figure 1B**).

Silence of TTN-AS1 inhibited cell migration and invasion in LCC9 BC cells

In this study, we chose LCC9 BC cell lines for the silence of TTN-AS1. Then TTN-AS1 expression was detected by RT-qPCR (**Figure 2A**). Moreover, results of wound healing assay showed that silence of TTN-AS1 significantly inhibited the ability of cell migration in BC cells (**Figure 2B**). The outcome of transwell assay also revealed that the number of migrated cells was remarkably decreased after TTN-AS1 was silenced in BC cells (**Figure 2C**). The number of invaded cells was remarkably decreased after TTN-AS1 was silenced in BC cells (**Figure 2D**).

Overexpression of TTN-AS1 promoted cell migration and invasion in SKBR3 BC cells

In this study, we chose SKBR3 BC cell lines for the overexpression of TTN-AS1. Then TTN-AS1 expression was detected by RT-qPCR (**Figure3A**). Moreover, results of wound healing assay showed that overexpression of TTN-AS1 significantly promoted the ability of cell migration in BC cells (**Figure 3B**). The outcome of transwell assay also revealed that the number of migrated cells was remarkably increased after TTN-AS1 was overexpressed in BC cells (**Figure 3C**). The outcome of transwell assay also revealed that the number of invaded cells was remarkably increased after TTN-AS1 was overexpressed in BC cells (**Figure 3D**).

The interaction between miR-140-5p and TTN-AS1 in BC

DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) was used to find the miRNAs that contained complementary base with TTN-AS1. We selected miR-140-5p as it contained binding area of TTN-AS1(**Figure 4A**). RT-qPCR results showed that miR-140-5p was upregulated in sh-TTN-AS1 group compared with control group (**Figure 4B**). Meanwhile, miR-140-5p was downregulated in TTN-AS1 group compared with empty vector group (**Figure 4C**). Furthermore, results of luciferase assay showed that luciferase activity was significantly reduced through co-transfection of TTN-AS1-WT and miR-140-5p, while no significant changes of luciferase activity were observed through co-transfection of TTN-AS1-MUT and miR-140-5p (**Figure 4D**). In addition, RIP assay identified that TTN-AS1 and miR-140-5p were significantly enriched in Ago2-containing beads compared to input group (**Figure 4E**).

Discussion

LncRNAs can regulate gene expression through multiple mechanisms, mostly depending on subcellular localization and the nature of molecular interactors (DNA, RNA, and proteins). The interaction between lncRNA–miRNA functional networks has drawn much attention recently. By targeting miR-873, lncRNA NRF modulates programmed necrosis and myocardial injury during ischemia and reperfusion[8]. Through negatively regulating miR-200b/a/429, lncRNA ILF3-AS1 enhances cell proliferation, cell migration and invasion in melanoma[9]. By acting as a molecular sponge for miR-200s, depletion of lncRNA ZEB1-AS1 significantly suppresses cell proliferation and call migration in osteosarcoma[10]. LncRNA PCAT-1 facilitates cell invasion and metastasis in hepatocellular carcinoma via miR-129-5p-HMGB1 signaling pathway by directly binding to miR-129-5p[11].

Researches have proved that altered expression of many lncRNAs are associated with the progression of BC closely. For example, downregulation of lncRNA snaR inhibits the proliferation, migration, and invasion of BC cells and may be a potential treatment for triple-negative BC[12]. LncRNA OR3A4 facilitates cell proliferation and cell migration in BC through inducing epithelial-mesenchymal transition[13]. LncRNA linc-ITGB1 functions as an oncogene in BC by inducing cell cycle arrest[14]. LncRNA CAMTA1 enhances cell proliferation and cell mobility in BC by targeting miR-20b[15].

TTN-AS1 is a novel lncRNA which has reported to promote cell proliferation and cell migration in cervical cancer via sponging miR-573[16]. In our study, TTN-AS1 was upregulated in BC tissues. Besides, silence of TTN-AS1 inhibited cell migration and invasion in BC cells, while overexpression of TTN-AS1 promoted cell migration and invasion in BC cells. Above results indicated that TTN-AS1 promoted metastasis of BC and might act as an oncogene.

To further identify the underlying mechanism of how TTN-AS1 affects BC cell migration and invasion, we predicted and picked miR-140-5p as the potential binding microRNAs of TTN-AS1 through bioinformatic analysis and experimental verification. MiR-140-5p is dysregulated in various tumors. In addition, miR-140-5p has been reported to serve as a tumor suppressor in some tumors. For example, miR-140-5p inhibits cell proliferation and cell migration in gastric cancer via regulation of YES1[17]. By targeting fibroblast growth factor 9 and transforming growth factor β receptor 1, miR-140-5p depresses tumor growth and metastasis in hepatocellular carcinoma[18]. MiR-140-5p suppresses tumor growth and cell metastasis in cervical cancer by targeting insulin like growth factor 2 mRNA binding protein 1[19]. Moreover, miR-140-5p has also been reported to inhibit the invasion and angiogenesis of BC by targeting vascular endothelial growth factor-A (VEGFA)[20].

In the present study, miR-140-5p expression could be upregulated after knockdown of TTN-AS1. Meanwhile, miR-140-5p expression could be downregulated after overexpression of TTN-AS1. Moreover, miR-140-5p could directly bind to TTN-AS1 through a luciferase assay. MiR-140-5p was significantly enriched by TTN-AS1 RIP assay. All the results above suggest that TTN-AS1 might promote metastasis of BC via sponging miR-140-5p.

Conclusion

Above data identified that TTN-AS1 was remarkably upregulated in BC patients. Besides, TTN-AS1 could facilitate cell migration and invasion in BC through sponging miR-140-5p. These findings suggest that TTN-AS1 may contribute to therapy for BC as a candidate target.

Declarations

Consent for publication

Yes

Availability of data and material

Yes if request.

Competing interests

no

Funding

no

Authors' contributions

Yusheng Li made this manuscript, and Fan Wang help revise this work.

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Ethics approval and consent to participate

The research has been carried out in accordance with the World Medicial Association Declaration of Helsinki. All subjects provided written informed consent.

Disclosure

All authors declare no conflict of interest.

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Figures

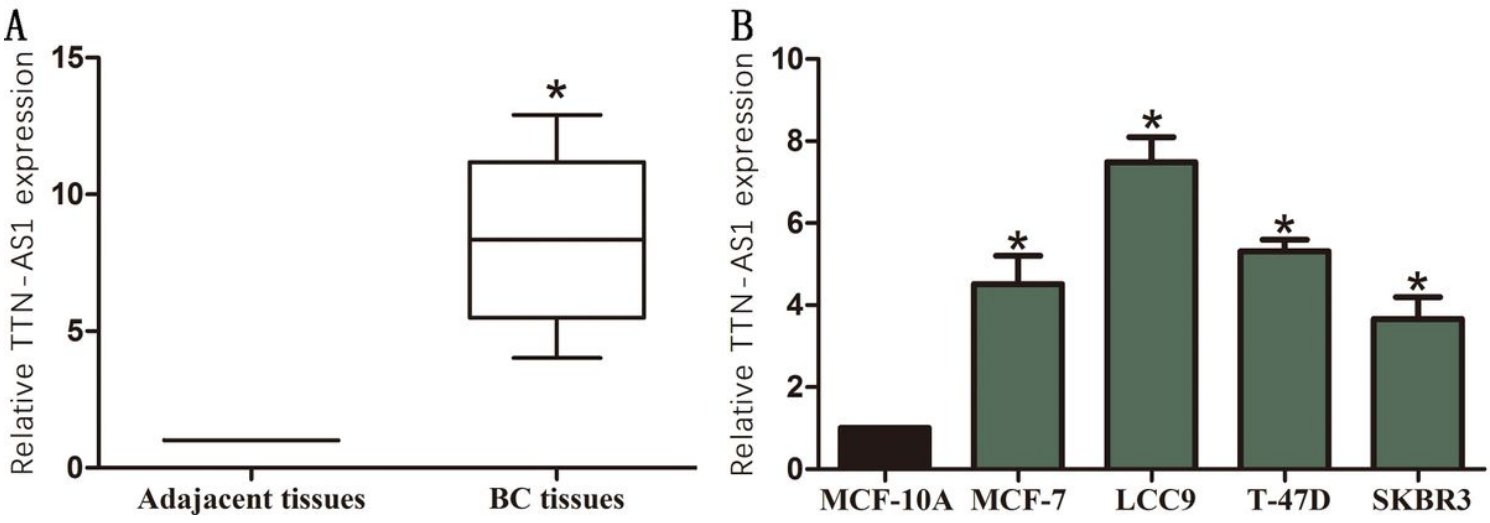

Figure 1

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 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.

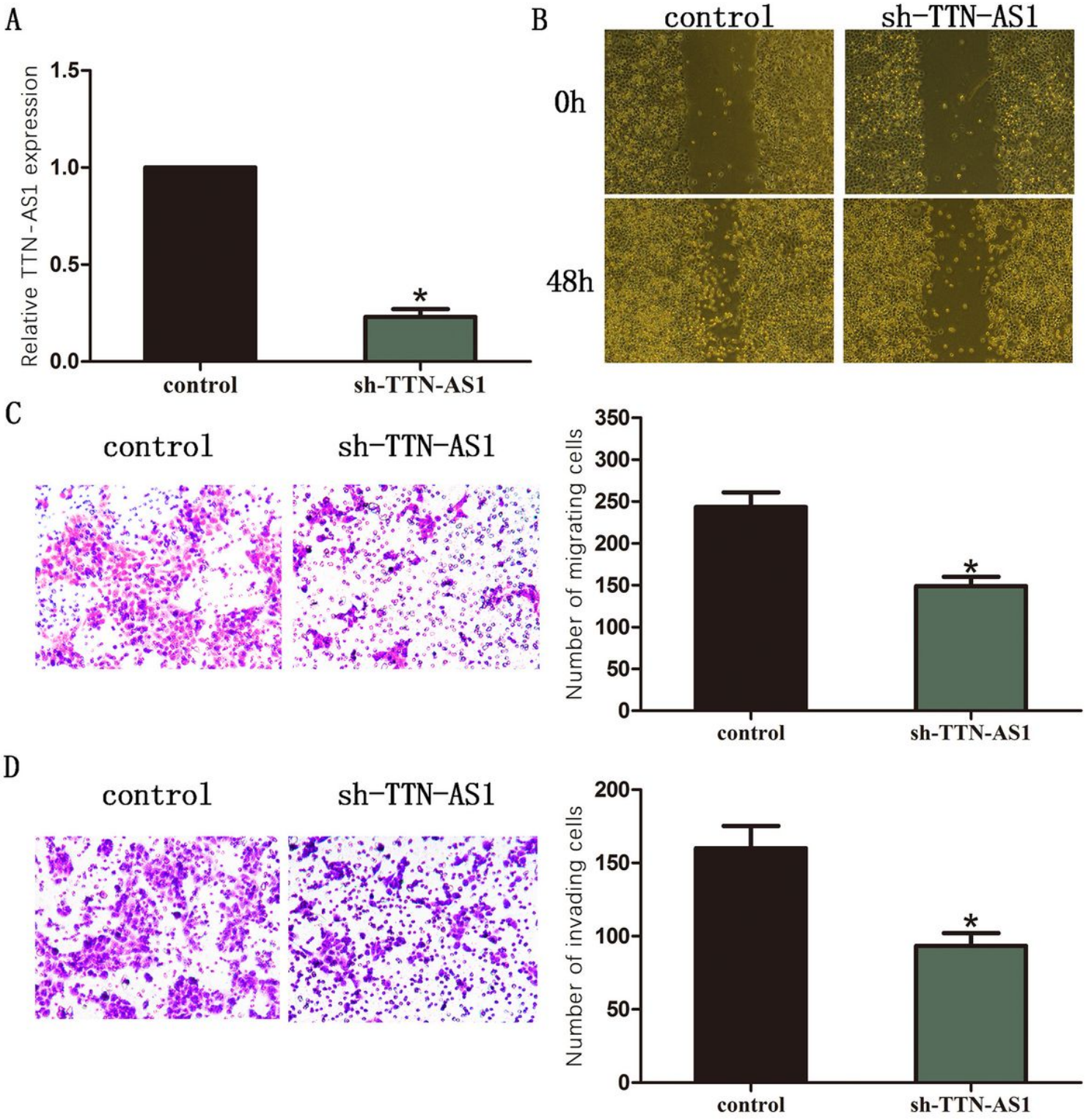

Figure 2

Silence of TTN-AS1 inhibited LCC9 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:10×). (C) Transwell assay showed that number of migrated cells was significantly decreased via silence of TTN-AS1 in BC cells

(magnification:40×). (D) Transwell assay showed that number of invaded cells was significantly decreased via silence of TTN-AS1 in BC cells (magnification:40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

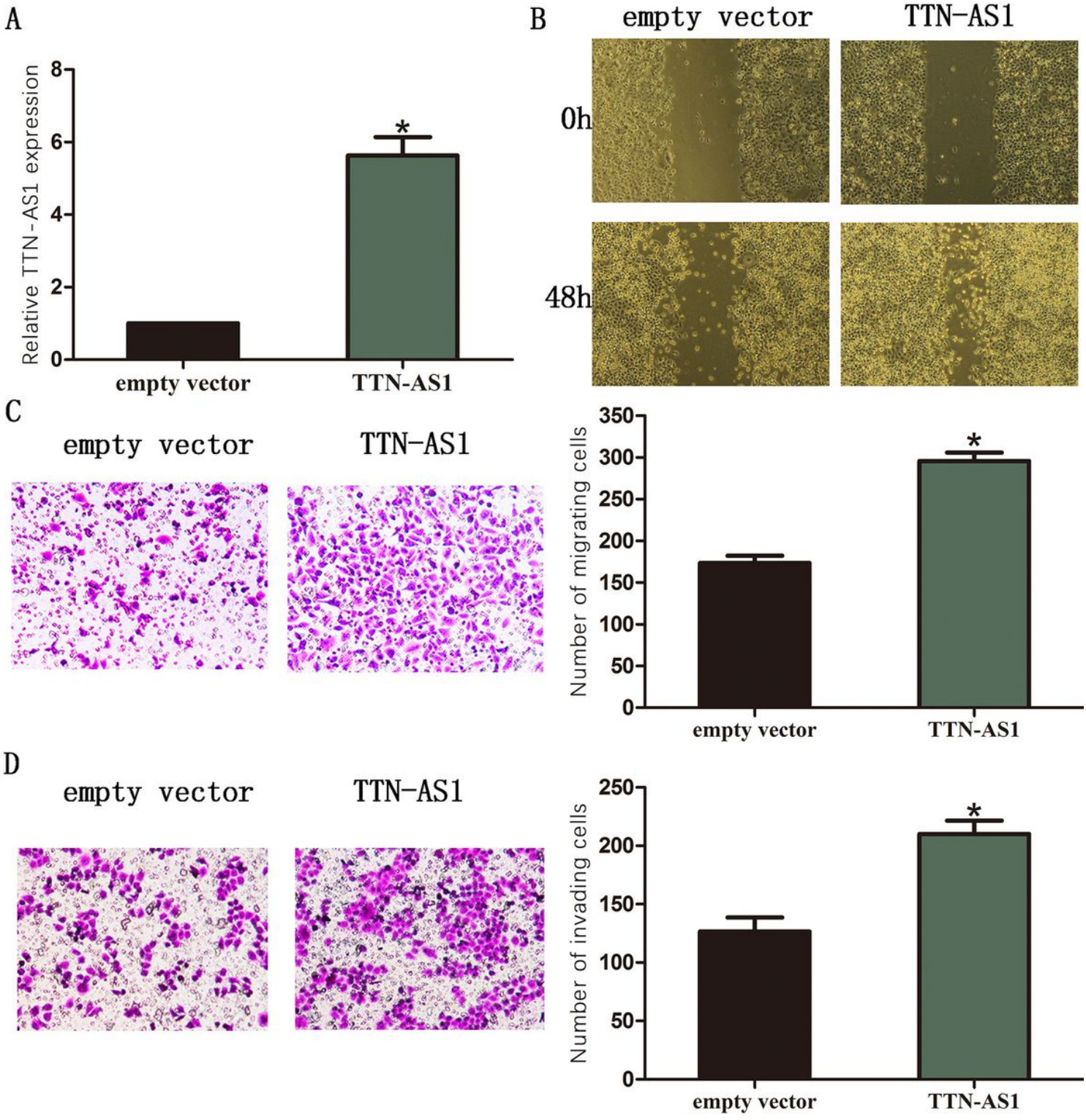

Figure 3

Overexpression of TTN-AS1 promoted SKBR3 BC cell proliferation and invasion. (A) TTN-AS1 expression in BC cells transduced with TTN-AS1 lentivirus (TTN-AS1) 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 TTN-AS1 significantly promoted cell migrated ability of BC cells(magnification:10×). (C) Transwell assay showed that number of migrated cells was significantly increased via overexpression of TTN-AS1 in BC cells(magnification:40×). (D) Transwell assay showed that number of invaded cells was significantly increased via overexpression of TTN-AS1 in BC cells(magnification:40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

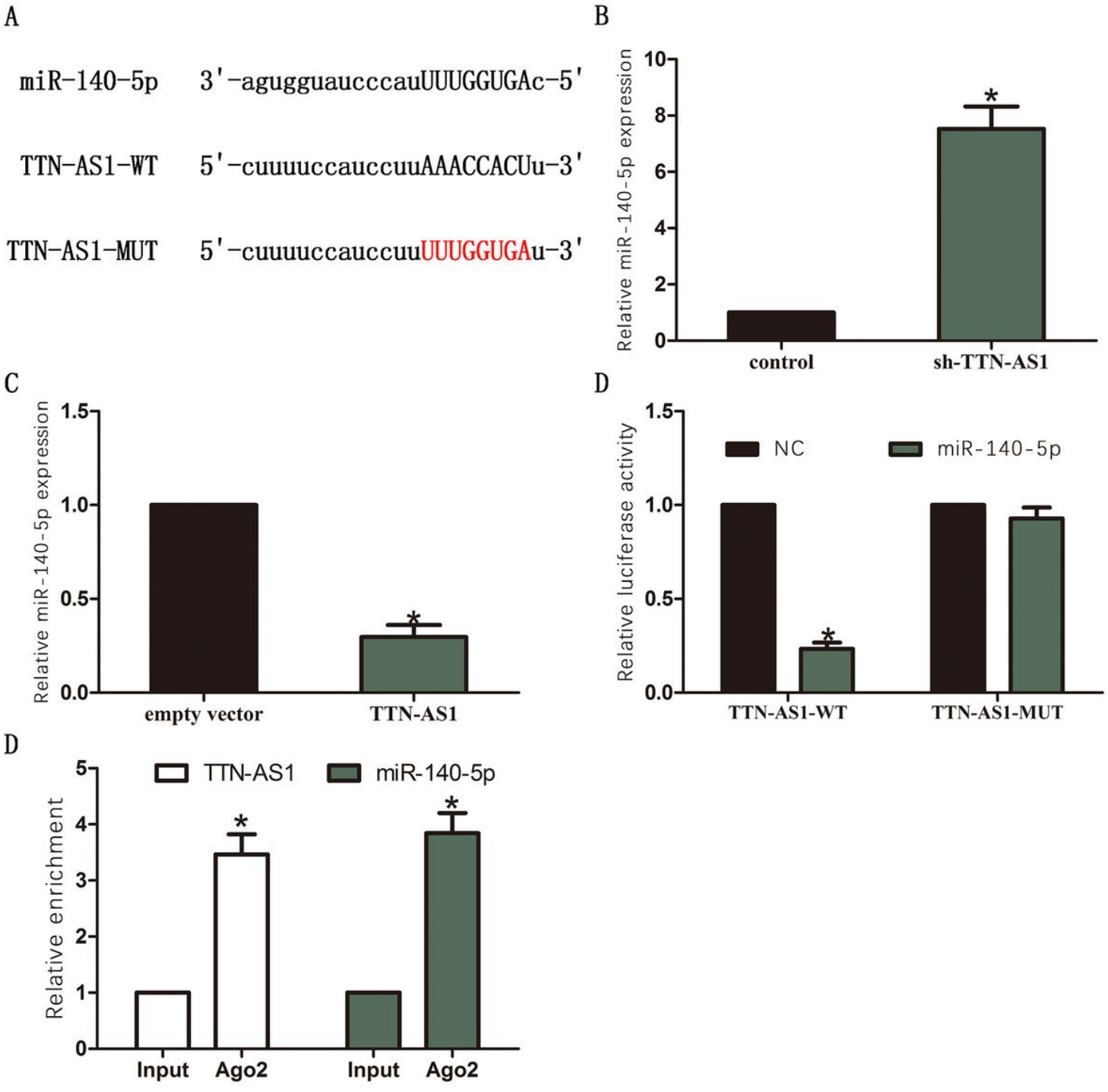

Figure 4

Association between TTN-AS1 and miR-140-5p in BC cells and tissues. (A) The binding sites of miR-140-5p on TTN-AS1. (B) The miR-140-5p expression was increased in sh-TTN-AS1 group compared with control group. (C) The miR-140-5p expression was decreased in TTN-AS1 group compared with empty vector group. (D) Co-transfection of miR-140-5p and TTN-AS1-WT strongly decreased the luciferase activity, while co-transfection of miR-140-5p and TTN-AS1-MUT did not change the luciferase activity. (E) RIP assay showed the enrichment of TTN-AS1 and miR-140-5p Ago2-containing beads. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.