Abstract. Increasing evidence has demonstrated that long non-coding RNAs (lncRNAs) serve important roles in numerous malignancies, including triple-negative breast cancer (TNBC). The lncRNA titin-antisense RNA1 (TTN-AS1) has previously been reported to promote tumorigenesis in various types of cancer. The present study aimed to investigate the potential role of TTN-AS1 in breast cancer and the associated underlying mechanisms. Following prediction by Starbase and confirmation by dual-luciferase reporter assay, TINCR was demonstrated to be a target gene for microRNA (miR)-211-5p. The expression levels of TTN-AS1 and miR-211-5p, which was predicted to be targeted by TTN-AS1, in TNBC tissues and in the breast cancer cell lines MDA-MB-453 and MDA-MB-231 were measured using reverse transcription-quantitative PCR. Following TTN-AS1-knockdown, cell proliferation was measured using a Cell Counting Kit-8 assay and colony formation assay, whereas cell invasion and migration were measured using Transwell and wound healing assays, respectively. Luciferase reporter assay was performed to verify the potential interaction between TTN-AS1 and miR-211-5p. In addition, rescue assays were conducted to investigate the effects of TTN-AS1 and miR-211-5p on TNBC development. The results demonstrated that TTN-AS1 expression was significantly upregulated, whereas that of miR-211-5p was found to be downregulated in TNBC tissues and cell lines compared with the matched adjacent normal tissues and normal breast epithelial cell line MCF-10A, respectively. Furthermore, TTN-AS1-knockdown inhibited the proliferation and invasive and migratory abilities of MDA-MB-453 and MDA-MB-231 cells, which was reversed following co-transfection with the miR-211-5p inhibitor. The results from luciferase reporter assay confirmed that miR-211-5p was a direct target of TTN-AS1, suggesting that TTN-AS1 may bind directly to miR-211-5p to negatively regulate its expression. In conclusion, the findings from the present study demonstrated that TTN-AS1 regulated the proliferation and invasive and migratory abilities of TNBC by targeting miR-211-5p. This study may provide some insights into the regulatory mechanism of TNBC and help the development of novel therapeutic interventions for TNBC.

Introduction

Breast cancer (BC) is one of the most common malignant tumors in women worldwide and is associated with high morbidity and mortality rates (1). It mostly occurs in women between the ages of 55 and 60 years (2). In 2019, ~268,600 new cases of BC were diagnosed and 41,760 women died from this disease in the US (3). Accumulating evidence suggests that the incidence of BC is increasing, and patients diagnosed with BC are younger (4,5). Triple-negative BC (TNBC) is a unique and aggressive subtype of BC, accounting for 15-20% of all cases of BC (6). In addition, TNBC is associated with high recurrence rate and poor outcomes (6). TNBC is characterized by the absence of the estrogen receptor, human epidermal growth factor receptor type 2 and progesterone receptor expression (7). Due to the lack of specific early diagnostic and prognostic biomarkers, the prognosis of TNBC remains poor (8). Therefore, an urgent demand currently exists for the development of possible diagnostic and therapeutic targets to improve the survival rates of patients with TNBC.

Long non-coding RNAs (lncRNAs) are RNA molecules that are >200 nucleotides in length and that do not encode proteins (9). Previous studies have suggested that lncRNAs can exert various functions in numerous biological processes, and that they are implicated in the development and progression of a variety of cancers (10,11). The lncRNA titin-antisense RNA1 (TTN-AS1) regulates the proliferation, invasion and migration of triple-negative breast cancer by targeting miR-211-5p

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(TTN-ASI) was first found to be a highly expressed oncogene in esophageal squamous cell carcinoma, where it can promote cell proliferation and metastasis (12). In addition, a previous study has reported that TTN-ASI increases the proliferation and invasive and migratory abilities of cervical cancer (13), whereas another study demonstrated that TTN-ASI can facilitate papillary thyroid cancer tumor progression (14). However, the role of TTN-ASI in TNBC remains poorly understood.

In the present study, clinical TNBC tissue samples and cell lines (MDA-MB-453 and MDA-MB-231) were used to investigate the biological function and underlying mechanism of TTN-ASI in TNBC. The results from the present study may uncover a novel therapeutic target for the intervention of TNBC.

Materials and methods

Clinical sample collection. A total of 30 pairs of TNBC and matched adjacent normal tissues (distance from tumor margin, 2 cm) were collected from patients with TNBC (age range, 27-79 years; mean age, 52.9±4.1 years) who underwent surgical resection at The Nanjing Maternal and Child Health Care Hospital (Nanjing, China) between April 2017 and May 2018. None of the patients received chemotherapy or radiotherapy prior to the operation. All resected tissues samples were immediately snap frozen in liquid nitrogen following surgery and stored at -80˚C for further analysis. The present study was approved by the Ethics Committee of Nanjing Maternal and Child Health Care Hospital and complied with the principles of the Helsinki Declaration. All patients provided signed informed consent.

Cell culture. The human TNBC cell lines MDA-MB-453 and MDA-MB-231 and the normal breast epithelial cell line MCF-10A were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and placed in a humidified atmosphere under 5% CO2 at 37˚C.

Cell transfection. MDA-MB-453 and MDA-MB-231 cells were seeded into 6-well plates at a density of 1x10^6 cells/well and transfection was performed when ~90% confluence was reached following manufacturer’s protocol. Short hairpin RNA (shRNA) targeted against TTN-ASI (shRNA-AS1) or shRNA-AS1 (shRNA-AS1-2) and a scrambled shRNA (shRNA) targeted against TTN-ASI (shRNA-AS1-1 or shRNA) were transfected into the TNBC cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and transfection was performed when ~90% confluence was reached. The colonies were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature, stained with 0.5% crystal violet for 10-30 min and imaged under a fluorescence inversion microscope (Olympus; magnification, 10x). ImageJ software (version 1.52r; National Institutes of Health) was used to count colonies.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 kit (Sigma-Aldrich; Merck KGaA) was used to detect cell proliferation. Briefly, transfected cells were seeded into 96-well plates (3,000 cells/100 µl). After incubation for 24, 48 or 72 h, 10 µl CCK-8 solution was added to each well and then cultured for a further 2 h. Absorbance was read at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. Cells in the logarithmic growth phase were transfected into 6-well plates at the density of 600 cells/well and were cultured at 37˚C for 14 days until colony formation became visible. Culture medium was regularly changed every 2 days. The colonies were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature, stained with 0.5% crystal violet for 10-30 min and imaged under a fluorescence inversion microscope (Olympus; magnification, 10x). Cell number was analyzed using ImageJ software (version 1.52r; National Institutes of Health).

Transwell assay. The invasive ability of TNBC cells was evaluated using Transwell assay (pore size, 8.0 µm; Corning Inc.) coated with Matrigel (BD Biosciences) overnight at 37˚C. Briefly, 500 µl serum-free media containing 2x10^5 cells were seeded in the upper chamber, and the lower chamber was filled with media containing 10% FBS. Invasive cells were stained with crystal violet 48 h after incubation, and were subsequently imaged under a fluorescence inversion microscope (Olympus; magnification, 100x). Cell number was counted using ImageJ software (version 1.52r; National Institutes of Health).

Wound healing assay. Cell migration was assessed using a wound healing assay. Briefly, cells (3x10^5 cells/well) were seeded into a 6-well plate and incubated in RPMI-1640 medium containing 10% FBS at 37˚C until 80% confluence was reached. Subsequently, cells were serum-starved for 24 h. A straight scratch was then introduced in the cell monolayers using a 200 µl plastic pipette tip. PBS was used to wash the cells and remove any debris. Following incubation for further 48 h in serum-free medium, the average distance of cells migrating into the wound was measured using an fluorescence inversion microscope (Olympus; magnification, 100x).

RNA extraction and RT-qPCR. Total RNA was isolated from TNBC tissue samples and cell lines using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc) according to the manufacturer’s protocol. PrimeScript® RT reagent Kit (Takara Bio, Inc.) was then used to produce the cDNA following manufacturer’s protocol. Subsequently, qPCR was performed using Power SYBR® Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Pre-denaturation at 95˚C for 10 min, denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min, for a total of 40 cycles. Sequences of the gene-specific primers used in this study were as follows: TTN-ASI forward, 5’-CGATAACATTGAAACAGCTG-3’ and reverse, 5’-GGTGAGGGGTCCAGTG-3’; miR-211-5p forward, 5’-ACACTCAGCGTGCAAGTAGCATACTA-3’ and reverse, 5’-AGTGTCGCTGGAGTTCG-3’; GAPDH forward, 5’-TGTTGGCGGCAATGGAATTGTTG-3’ and reverse, 5’-ACACATGATTCTCAGGTCACAT-3’; and U6 forward, 5’-TCTGGTCTCTATCCCAATTACCTG-3’ and reverse, 5’-ACTCCGGAACCCCTCTCTCTGAATG-3’ which were all synthesized by Guangzhou RiboBio Co., Ltd.
Pearson's correlation coefficients analysis was performed to compare TTN-B tissues compared with adjacent tissues (Fig. 1A and B). The expression of miR-211-5p was significantly decreased in TNBC tissues. The results demonstrate that TTN-AS1 expression was significantly increased, whereas miR-211-5p expression was negatively correlated with TTN-AS1 expression in TNBC tissues. In addition, significantly higher expression of TTN-AS1 and lower miR-211-5p expression were observed in MDA-MB-453 and MDA-MB-231 cells compared with those in MCF-10A cells (Fig. 1E and F). These results indicated that TTN-AS1 expression was upregulated and miR-211-5p expression was downregulated in TNBC tissues and TNBC cells compared with controls.

**Western blotting.** Total protein in TNBC tissue samples and cell lines was isolated using RIPA reagent buffer (Beyotime Institute of Biotechnology) at 4°C, and the bicinechonic acid protein assay kit (Beyotime Institute of Biotechnology) was used to determine protein concentration. Proteins (40 µg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5% skimmed milk and incubated with specific primary antibodies overnight at 4°C. Primary antibodies included the anti-matrix metalloproteinase 2 (MMP2) (cat. no. 40994S; 1:1000), anti-MMP9 (cat. no. 13667T; 1:1000), all from Cell Signaling Technology, Inc. After rinsing with TBS-0.2% Tween-20 three times, the membranes were incubated with a goat anti-rabbit HRP-conjugated secondary antibody (cat. no. 7074S; 1:3000; Cell Signaling Technology, Inc.) at room temperature for 1.5 h. The blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences) and subsequently quantified using ImageJ software (version 1.52r; National Institutes of Health).

**Luciferase reporter assay.** Potential target genes of IncRNA TTN-AS1 were predicted using an online bioinformatics software (http://starbase.sysu.edu.cn/) (17,18), and the potential interaction between miR-211-5p and TTN-AS1 was determined using luciferase reporter assay. Luciferase reporter vectors encoding the wild-type (WT) or mutant (MUT) TTN-AS1 3'-untranslated region (3'-UTR) were first designed. To perform the luciferase reporter assay, cells were first co-transfected with TTN-AS1 3'-UTR-WT or TTN-AS1 3'-UTR-MUT and miR-211-5p mimic or miR-NC, following which luciferase activity was detected using a Dual-Luciferase® Reporter Assay System (Promega Corporation) 48 h after transfection. Renilla luciferase activity was used as control.

**Statistical analysis.** All data were presented as the means ± standard deviation and analyzed using GraphPad Prism 6 (GraphPad Software, Inc.). Pearson's correlation coefficient was applied to assess the correlation between the expression of TTN-AS1 and miR-211-5p in TNBC tissues and adjacent normal tissues. A paired t-test was used for comparisons between TNBC and matched adjacent tissues. One-way analysis of variance followed by Tukey's post hoc test was used to evaluate differences among different groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

TTN-AS1 expression is upregulated, whereas miR-211-5p expression is downregulated in TNBC tissues and TNBC cells. RT-qPCR was performed to evaluate TTN-AS1 and miR-211-5p expression in human TNBC tissues. The results demonstrate that TTN-AS1 expression was significantly increased, whereas the expression of miR-211-5p was significantly decreased in TNBC tissues compared with adjacent tissues (Fig. 1A and B). Pearson's correlation coefficients analysis was performed to determine the correlation between TTN-AS1 and miR-211-5p expression in matched adjacent tissues and TNBC tissues. The results demonstrated that there was no correlation between TTN-AS1 and miR-211-5p expression in normal tissues, whereas miR-211-5p expression was negatively correlated with TTN-AS1 expression in TNBC tissues (F=0.3157; P<0.05; Fig. 1C and D). In addition, significantly higher expression of TTN-AS1 and lower miR-211-5p expression were observed in MDA-MB-453 and MDA-MB-231 cells compared with those in MCF-10A cells (Fig. 1E and F). These results indicated that TTN-AS1 expression was upregulated and miR-211-5p expression was downregulated in TNBC tissues and TNBC cells compared with controls.

TTN-AS1 knockdown increases the expression of miR-211-5p in TNBC cells. To investigate the regulatory relationship between TTN-AS1 and miR-211-5p, TTN-AS1 shRNA-1 and shRNA-2 were transfected into both MDA-MB-453 and MDA-MB-231 cells to knockdown TTN-AS1 expression. As presented in Fig. 2A and B, the expression levels of TTN-AS1 in both MDA-MB-453 and MDA-MB-231 cells were significantly decreased following transfection with shRNA compared with shRNA-NC. Since transfection with shRNA-1 exhibited the greater silencing effect, TTN-AS1 shRNA-1 was used for subsequent experiments. The expression of miR-211-5p was first evaluated following TTN-AS1 knockdown. The depletion of TTN-AS1 expression induced the upregulation of miR-211-5p in MDA-MB-453 and MDA-MB-231 cells (Fig. 2C and D). These data suggested that TTN-AS1 may affect the expression of miR-211-5p in TNBC cells.

miR-211-5p is a direct target of TTN-AS1. Using an online bioinformatics program (http://starbase.sysu.edu.cn/), it was found that miR-211-5p was a potential target of TTN-AS1 (Fig. 3A). Then, miR-211-5p was overexpressed by transfection with miR-211-5p mimic. As presented in Fig. 3B and C, the expression level of miR-211-5p was significantly upregulated in miR-211-5p mimic group compared with the miR-NC group. Subsequently, dual-luciferase assay was used to verify this potential relationship. Luciferase activity was found to be decreased following co-transfection with the miR-211-5p mimic and IncRNA TTN-AS1-WT (Fig. 3D and E), suggesting that miR-211-5p may be a direct target of TTN-AS1.

Transfection with the miR-211-5p inhibitor reverses the inhibitory impact of TTN-AS1 knockdown on TNBC cell proliferation. To explore the regulatory relationship between TTN-AS1 and miR-211-5p in TNBC, a miR-211-5p inhibitor was transfected into MDA-MB-453 and MDA-MB-231 cells, and RT-qPCR was performed to examine the transfection efficiency. miR-211-5p expression was significantly downregulated following transfection with the miR-211-5p inhibitor in the two TNBC cell lines (Fig. 4A and B). Cell proliferation was then measured using CCK-8 (Fig. 4C and D) and colony formation (Fig. 4E and F) assays. The results demonstrated that depletion of TTN-AS1 levels decreased the cell viability and colony numbers of both MDA-MB-453 and MDA-MB-231 cells compared with the shRNA-NC group, which was reversed by transfection with the miR-211-5p inhibitor. These findings suggested that TTN-AS1 may regulate TNBC cell proliferation by targeting miR-211-5p.
Transfection with the miR-211-5p inhibitor inhibits the effects of TTN-AS1 knockdown on the migratory and invasive abilities of TNBC cells. To understand the role of TTN-AS1 in invasion and migration, Transwell and wound healing assays were performed with TNBC cells. The results of the wound healing assay demonstrated that transfection with the TTN-AS1 shRNA-1 decreased MDA-MB-453 cell migratory ability compared with cells transfected with negative control. Subsequently, the migratory ability was recovered following co-transfection with the miR-211-5p inhibitor (Fig. 5A and B). Similar results were observed from the cell invasion assays (Fig. 5C and D). In addition, changes in cell migratory (Fig. 6A and B) and invasive (Fig. 6C and D) abilities in the MDA-MB-231 cell line following transfection with TTN-AS1 shRNA-1 and/or the miR-211-5p inhibitor presented similar results as those obtained with MDA-MB-453 cells.

Subsequently, the protein expression of matrix metalloproteinase (MMP)-2 and MMP-9, which are associated with migration, were assessed by western blotting. As presented in Fig. 7A and B, TTN-AS1 knockdown significantly downregulated the expression of MMP-2 and MMP-9 compared with cells transfected with shRNA-NC, which was reversed by co-transfection with the miR-211-5p inhibitor. These observations suggested that TTN-AS1 may regulate TNBC cell invasive and migratory abilities by targeting miR-211-5p.

Discussion

Accumulating evidence has demonstrated that IncRNAs participate in the tumorigenesis process. For instance, IncRNA long intergenic non-protein coding RNA, p53 induced transcript...
Figure 2. TTN-AS1 knockdown increased miR-211-5p expression in TNBC cells. Expression levels of TTN-AS1 in (A) MDA-MB-453 and (B) MDA-MB-231 cells were determined by RT-qPCR following transfection with shRNA-TTN-AS1-1 or shRNA-TTN-AS1-2. ***P<0.001 vs. shRNA-NC. Expression levels of miR-211-5p were measured in (C) MDA-MB-453 and (D) MDA-MB-231 cells using RT-qPCR following TTN-AS1 knockdown. *P<0.05 and **P<0.01 vs. shRNA-NC. NC, negative control; TTN-AS1, titin-antisense RNA1; TNBC, triple-negative breast cancer; miR, microRNA; shRNA, short hairpin RNA; RT-qPCR, reverse transcription-quantitative PCR.

Figure 3. miR-211-5p was a direct target of TTN-AS1. (A) Predicted binding region between TTN-AS1 and miR-211-5p. Expression of miR-211-5p was examined using reverse transcription-quantitative PCR in (B) MDA-MB-453 and (C) MDA-MB-231 cells. ***P<0.001 vs. miR-NC. Relative luciferase activities were measured in (D) MDA-MB-453 and (E) MDA-MB-231 cells. ***P<0.001 vs. TTN-AS1 + miR-NC. TTN-AS1, titin-antisense RNA1; miR, microRNA; NC, negative control.
was reported to suppress miR-523-3p and hamper retinoblastoma progression by upregulating Dickkopf-1 (19). IncRNA differentiation antagonizing non-protein coding RNA promotes the growth and metastasis of oral squamous cell carcinoma cells.
by altering miR-216a-5p expression (20). In addition, lncRNAs have been reported to serve as pivotal regulators in the initiation and progression of a number of malignancies, including TNBC (21-23). The lncRNA TTN-AS1 is a newly identified lncRNA; the increased expression of which was first reported in esophageal squamous cell carcinoma (12). TTN-AS1

![Figure 5.](image-url)

Figure 5. miR-211-5p inhibitor attenuated the effects of TTN-AS1 knockdown on MDA-MB-453 cell migratory and invasive abilities. (A) Cell migration was measured using wound healing assay. (B) Quantification of wound healing assay data. (C) Representative images and (D) relative quantification of cell invasion, as measured using Transwell assay. **P<0.01 vs. shRNA-NC; ###P<0.001 vs. shRNA-TTN-AS1-1 + miR-NC. NC, negative control; TTN-AS1, titin-antisense RNA1; miR, microRNA; shRNA, short hairpin RNA.
has been demonstrated to accelerate the proliferation and invasive and migratory abilities of cervical cancer cells, in addition to facilitating the progression of papillary thyroid cancer tumors (13,14). The present study demonstrated that TTN-ASI was overexpressed in TNBC tissues and cell lines compared with normal adjacent tissues and the normal breast epithelial cell line, respectively. In addition, TTN-ASI knockdown inhibited the proliferation and invasive and migratory abilities of TNBC cells by targeting miR-211-5p expression, which may provide a potentially novel insight into the development of therapeutic strategies for clinical intervention of TNBC.
It has been reported that lncRNAs can modulate gene expression by sponging specific miRNAs, indirectly regulating gene expression at the post-transcriptional level (24). Therefore, bioinformatics predictions were performed using the TargetScan online tool, which predicted miR-211-5p to be a direct target of TTN-AS1. This was verified using luciferase reporter assays in the present study. Increasing evidence suggested that miR-211-5p functions as a tumor suppressor in numerous malignancies, including renal cell carcinoma, papillary thyroid cancer and tongue cancer (15,25,26).

Notably, emerging evidence supports the notion that miR-211-5p expression is downregulated in TNBC tissues and cells, and the upregulation of miR-211-5p expression can inhibit the progression of TNBC (27). To understand the regulatory relationship between TTN-AS1 and miR-211-5p, TTN-AS1 shRNA was transfected into MDA-MB-453 and MDA-MB-231 cells, following which TNBC cell proliferation was measured using CCK-8 and colony formation assays. It was found that TTN-AS1 knockdown inhibited the proliferation of TNBC cells, which was reversed following transfection with miR-211-5p. Excessive proliferation of cancer cells can accelerate the progression of tumorigenesis and development (28). These findings suggested therefore that TTN-AS1 may regulate TNBC proliferation by targeting miR-211-5p.

Cancer cell migration and invasion are pivotal processes of metastasis, which is the primary cause of cancer-associated mortality (29). During cancer development, the migratory and invasive abilities of cancer cells are increased, resulting in cancer cell migration to distant sites throughout the body, where they can become secondary tumors (30). It has previously been suggested that the poor prognosis associated with TNBC is associated with enhanced invasive and migratory abilities of TNBC cells (31). The present study demonstrated that TTN-AS1 knockdown inhibited the invasive and migratory abilities of both MDA-MB-453 and MDA-MB-231 cells, which was reversed by transfection with the miR-211-5p inhibitor. MMPs, including MMP-2 and MMP-9, are major components that are implicated in invasion and migration, and the upregulation of these proteins is closely associated with the aggressiveness and metastasis of TNBC (32-34). In the present study, decreased MMP-2 and MMP-9 expression was observed following TTN-AS1 knockdown, which was reversed following transfection with the miR-211-5p inhibitor. These findings suggested that TTN-AS1 may regulate TNBC invasive and migratory abilities by targeting miR-211-5p.

The present study investigated the role of TTN-AS1 in TNBC and the potential underlying mechanisms. TTN-AS1 was found to be significantly upregulated in TNBC tissues and cell lines, suggesting that TTN-AS1 may be considered as a significant biomarker in the evaluation of patients with...
TNBC prognosis. In addition, TTN-AS1 was found to regulate TNBC proliferation and invasive and migratory abilities by targeting miR-211-5p, which may provide novel insights into the mechanism of TNBC and help the development of novel therapeutic interventions.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ES and XL interpreted the data and performed the experiments. CL and KL collected the data, searched the literature and designed the study. ES wrote the manuscript and KL revised the manuscript. All authors read and approval the final manuscript.

Ethics approval and consent to participate

All patients in this study signed the written informed consents. This study was approved by the Ethics Committee of Nanjing Maternal and Child Health Care Hospital and complied with the principles of the Helsinki Declaration.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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