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Long noncoding RNA TTN-AS1 promotes the proliferation and migration of prostate cancer by inhibiting miR-1271 level

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Abstract. – OBJECTIVE: Prostate cancer is one of the most common malignant tumors. Recently, the role of long noncoding RNAs (lncRNAs) in tumor progression has been well concerned by numerous researchers. In this research, lncRNA TTN-AS1 was studied to identify its biological function in the progression of prostate cancer.

PATIENTS AND METHODS: Firstly, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was conducted to measure TTN-AS1 expression in prostate cancer tissues. Furthermore, *in vitro* role of TTN-AS1 in regulating prostate cancer cells was assessed. Tum mation assay was conducted in NOD/SC to explore the *in vitro* function of TTN-A In addition, the luciferase reporter gene assay performed to analyze the relationship betw TTN-AS1 and miR-1271.

RESULTS: TTN-AS1 expres remark ably higher in prostate ca s com pared with that of corres ing o . More over, proliferation and ration tate cancer cells were inhibit er silenced. MiR-1271 up d after the silence of TTN-AS rther m ism assays showed that mi 1 was a d arget of TTN-AS1 in pr ncer. In add , tumor formation an etas abilities were inhibited after *in* knockd TTN-AS1.

CONCL ONS: Our stu covers a potential on ne in prostate ca er and demonstrate at TTN S1 enhances cell proliferation and ration sponging miR-1271.

Key w
ong ding TTN-AS1, Prostate cancer, 71.

Introduction

Prostate cancer is reported to kill more than 2 men in the United States and leads to 13% of cancer-related death in 2018[1]. The surgery or androgen deprivation therapy is available for most of the newl osed prosta er patients at an earl g wever, the c currence and recurrence ates o ate cancer significantly increa both devel nd developing countri n makes it the rd-leading cause of d s in male malignancies all over the world[2-5]. efore, it is nt to find out the underlying hanism of pa genesis of prostate cancer and fi ut a new tment strategy.

ng RNAs (lncRNAs) are one subgroup on-coding RNAs. Recent evidence has proved that lncRNAs act as a vital role in the on of malignant tumors. For example, the lncRNA SNHG16 induces cell apoptosis, inhibits cell proliferation, and migration in papillary thyroid cancer[6]. LncRNA PCAT-1 modulates TP53-miR-215-PCAT-1-CRKL axis and has a vital function in tumorigenesis of hepatocellular carcinoma[7]. LncRNA DLEU7-AS1 promotes the occurrence and development of colorectal cancer Wnt/β-catenin pathway[8]. The knockdown of lncRNA SNHG5 inhibits malignant cellular phenotypes of glioma via Wnt/CTNNB1 signaling pathway [9].

LncRNA TTN-AS1 is a novel lncRNA, and its function in cancers has been focused on. Our study aimed to identify whether TTN-AS1 participates in the proliferation and migration of prostate cancer and its potential mechanism.

Patients and Methods

Tissue Specimens

58 prostate cancer patients received surgery at Baoji Central Hospital. Their tissue samples were surgically resected and immediately preserved at –80°C. All tissues were analyzed by an experienced pathologist. The Research Ethics Committee of Baoji Central Hospital ratified this study and the written informed consent was offered by the patients.

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Cell Culture

The human prostate cancer cell lines (22Rv1, DU145, and LNCaP) and normal human prostate epithelial cell line (P69) were offered by the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). The cell culture was conducted in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and 1% penicillin. Besides, the cells were cultured in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

shRNAs directed against TTN-AS1 were synthesized by GenePharma (Shanghai, China). The complementary DNA encoding TTN-AS1 was amplified and inserted into pcDNA3.1 (GenePharma, Shanghai, China), which were then used for the transfection of 22Rv1 cells with polybrene (Genepharma, Shanghai, China).

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA from tissues and cel extracted by using TRIzol reagent (In gen, Carlsbad, CA, USA). RNA was reve transcribed to cDNAs through reverse Tr scription Kit (TaKaRa Biotechn Co., Lt Dalian, China). Thermocycli ns were as follows: pre-denaturati 95°C 5 min, denaturation at 95°C fo s and a g at 60°C for 30 s, for a tota yc method was utiliz or c ng relative expression. The p er sequen re as follows: TTN-AS1 rd: 5'-TCC GCAT-CACCTAGC a erse: 5'-GA GGAG-GAAGTAG CATT glyceraldehyde 3-phosph dehydrogen GAPDH), forward: GCAC CGTCAA CTGAGAAC-3' and erse: GGTGAAGACGCCAGTG-GA

Cell Proliferation

10^3 transf d cells were seeded in 96-well pla d cell proliferation was assessed by Cell P eagent Kit I (MTT; Roche, Basel, itzerland) at 0 h, 24 h, 48 h, 72 h following the ufacturer's protocol. Absorbance at 490 nm w essed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay

1.5×10^3 22Rv1 cells per well were inoculated in a 6-well plate. 10 days later, the colo then fixed with 10% formaldehyde and stained for 5 min with 0.5% c violet. A Nikon camera (Tokyo, Japan) wa d for taking photographs of colonies.

Ethynyl Deoxyuridine (EdU) Incorporation Assay

The EdU Kit (Ro Basel tzerland) was used for detecting ce ifer n. A re entative photograph w ake ss Ax t Photomicroscope (Zeiss, Ob rmany).

Transwell Assay

24 h a er tra n, 2 $\times10^5$ cells in 100 µL se free DME e transformed to the to er of an 8-µm ure insert (Corning, C ng, NY, USA). 20% FBS-DMEM was added to the botto amber of the culture inserts. later, these erts were fixed by methanol f min and s ed by hematoxylin for 20 min. An oscope (×40) was utilized for counting atory cells in three random fields.

Luciferase Reporter Gene Assay

-UTR of TTN-AS1 was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3'-UTR. The site-direction mutagenesis of the miR-1271 binding site in TTN-AS1 3'-UTR as mutant (MUT) 3'-UTR was conducted through quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). After co-transfection, the luciferase assay was conducted on the dual-luciferase reporter gene assay system (Promega, Madison, WI, USA).

Tumor Formation Assay

After transfection, the 22Rv1 cells (6×10^5/mL) were injected into NOD/SCID mice (6 weeks old) subcutaneously. Tumor diameter was detected every 5 days. Tumor volume was calculated as the formula (volume = length × width^2 × 1/2). The tumors were extracted after 4 weeks.

Tumor Metastasis Assay

The transfected 22Rv1cells were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed, and the lungs were extracted after 4 weeks. Then the number of metastatic nodules in the lung was counted. The research was approved by the Animal Ethics Committee of Baoji Central Hospital.

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Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 5.0 (La Jolla, CA, USA). The difference between the two groups was compared by the Student's *t*-test. The statistical significance was defined as $p<0.05$.

Results

TTN-AS1 Expression Level in Prostate Cancer Tissues and Cells

First, RT-qPCR was conducted for detecting TTN-AS1 expression in 58 prostate cancer tissues and 3 prostate cancer cell lines. As a result, TTN-AS1 was significantly upregulated in tumor tissue samples (Figure 1A). TTN-AS1 level in prostate cancer cells was higher than that of P69 (Figure 1B).

Knockdown of TTN-AS1 Inhibited Cell Proliferation and Migration in DU145 Cells

In our study, we chose the 22Rv1 cell line for establishing the knockdown model of TTN-AS1. Then, the RT-qPCR was performed for verifying the transfection efficacy (Figure 2A). MTT assay showed that the cell growth ability of 22Rv1 cells was significantly repressed after TTN-AS1 knockdown (Figure 2B). The colony formation assay showed that the colony number was significantly reduced after knockdown of TTN-AS1 (Figure 2C). Furthermore, EdU incorporation assay also showed that the number of EdU positive cells was reduced after knockdown of TTN-AS1 in 22Rv1 cells (Figure 2D). The transwell assay showed that the number of migratory cells was significantly reduced after TTN-AS1 knocked down (Figure 2E).

The Interaction between MiR-1271 and TTN-AS1 in Prostate Cancer

Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to find the lncRNA that contained a complementary base with TTN-AS1. Because miR-1271 was a tumor suppressor and was able to suppress cell proliferation, we focused on miR-1271 binding these lncRNAs which were interacted with TTN-AS1 (Figure 3A). Indeed, RT-qPCR assay showed that the expression of miR-1271 was higher in TTN-AS1/shRNA cells than that in the control cells (Figure 3B). Furthermore, the dual-luciferase reporter gene assay revealed that the co-transfection of TTN-AS1-WT and miR-1271 mimics largely decreased the luciferase activity, while the co-transfection of TTN-AS1-MUT and miR-1271 mimics had no effect on the luciferase activity (Figure 3C). Meanwhile, the expression of TTN-AS1 was negatively correlated with miR-1271 in prostate cancer tissues (Figure 3D). In summary, these data demonstrated that miR-1271 was a direct target of TTN-AS1.

TTN-AS1 Knockdown Inhibited Tumor Formation and Metastasis in vivo

Next, *in vivo* effect of TTN-AS1 on tumor formation was detected. The tumor size in TTN-AS1/shRNA group was smaller compared with the empty vector (CONTROL) group (Figure 4A). The number of metastatic nodules in the lung

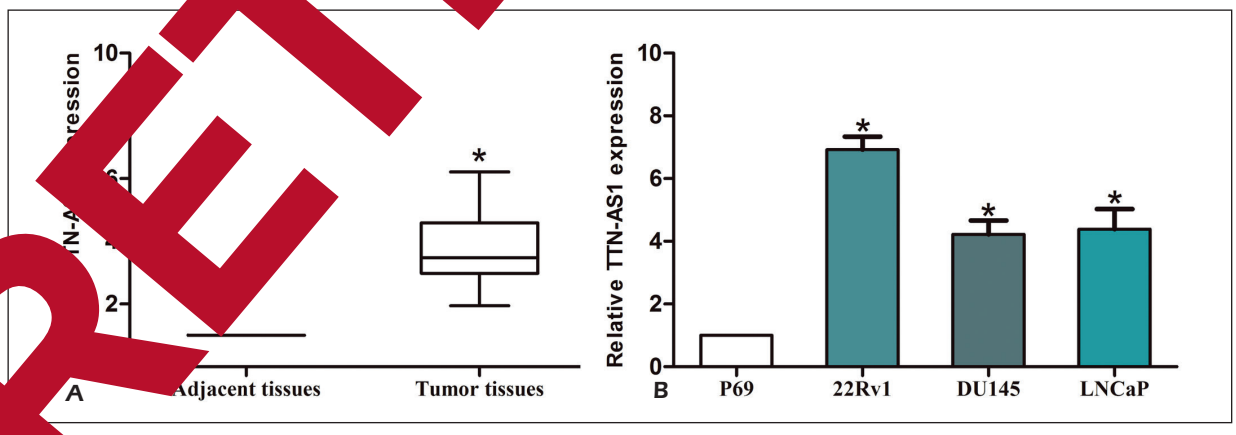

Figure 1. The expression levels of TTN-AS1 were upregulated in prostate cancer tissues and cell lines. **A**, TTN-AS1 expression was significantly upregulated in the prostate cancer tissues compared with adjacent tissues. **B**, The expression levels of TTN-AS1 relative to GAPDH were determined in the human prostate cancer cell lines and P69 (normal human prostate epithelial cell lines) by RT-qPCR. The data are presented as the mean ± standard error of the mean. *$p<0.05$.

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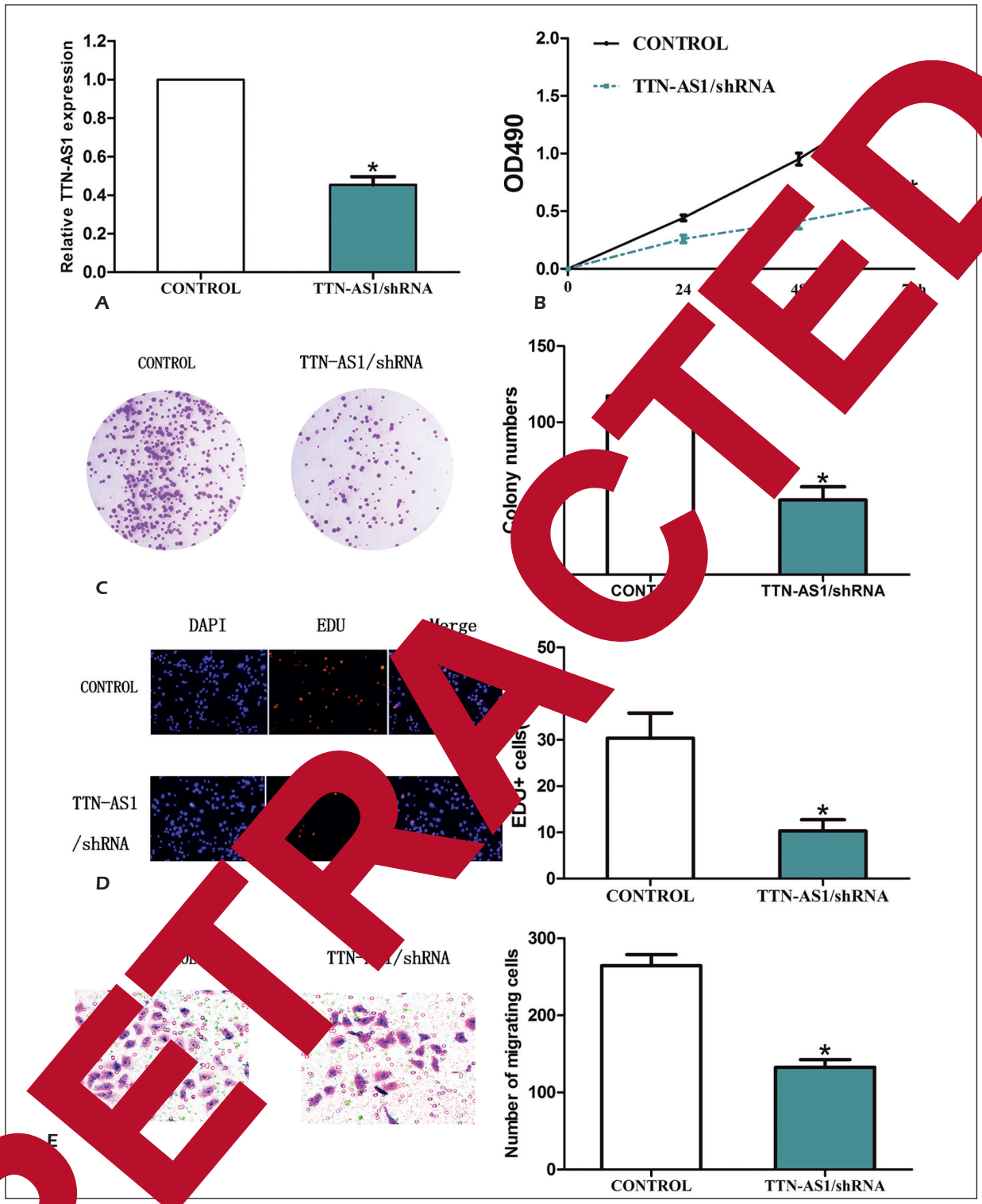

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Fig knockdown of TTN-AS1 inhibited proliferation and migration of 22Rv1 cells. **A**, TTN-AS1 expression in 22Rv1 c ith TTN-AS1 shRNA (TTN-AS1/shRNA) or the empty vector (CONTROL) was detected by RT-qPCR. GAP- was used as an internal control. **B**, MTT assay showed that the knockdown of TTN-AS1 significantly inhibited cell growth Rv1 cells. **C**, Colony formation assay showed that the number of colonies was reduced after the knockdown of TTN-AS1 in ells (magnification: 10×). **D**, EdU incorporation assay showed that EdU-positive cells were reduced after the knockdown of S1 in 22Rv1 cells. **E**, The transwell assay showed that the number of migratory cells was reduced after the knockdown of TTN-AS1 in 22Rv1 cells (magnification: 40×). The results represent the average of the three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

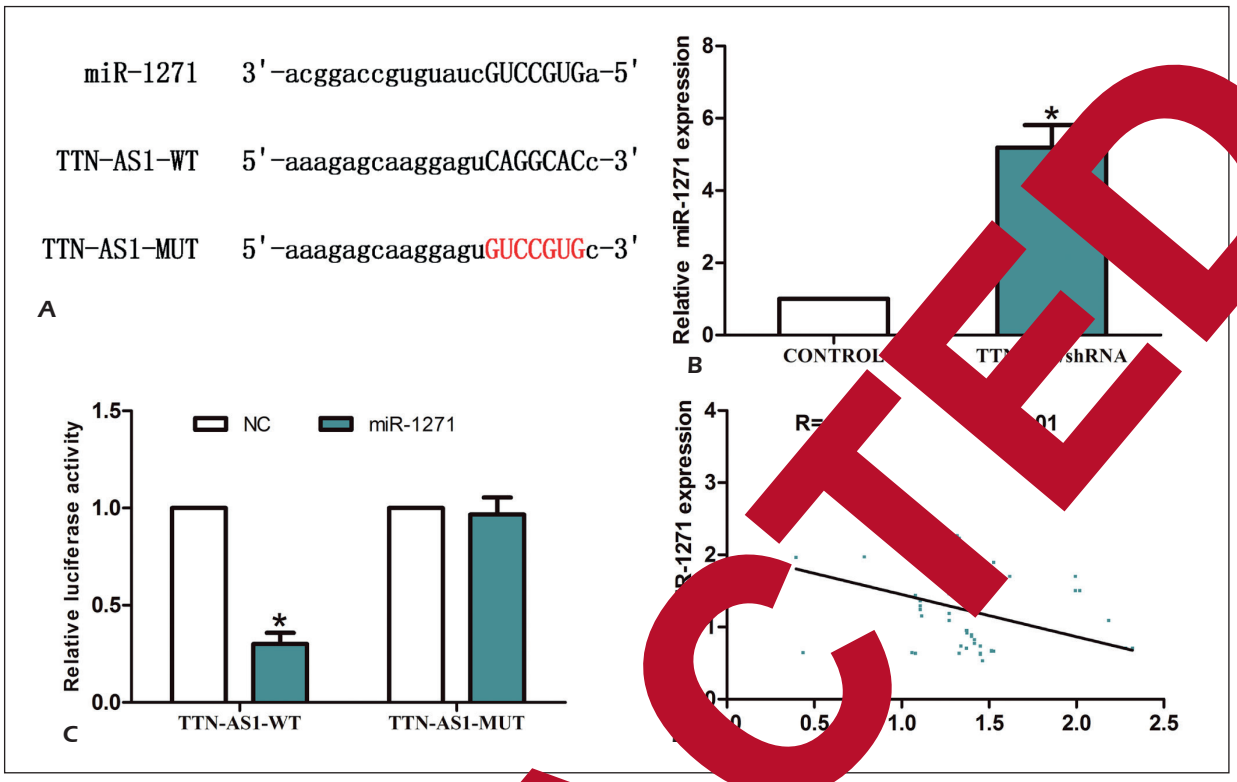

Figure 3. Reciprocal repression between TTN-AS1 and miR-1271. **A**, The binding sites of miR-1271 on TTN-AS1. **B**, MiR-1271 was upregulated in TTN-AS1/shRNA group compared with the empty vector (CONTROL) group. **C**, Co-transfection of miR-1271 and TTN-AS1-WT strongly decreased the luciferase activity, while co-transfection of the negative control (NC) and TTN-AS1-WT did not change the luciferase activity. **D**, The linear relationship between the expression levels of TTN-AS1 and miR-1271 in prostate cancer tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

from the TTN-AS1/shRNA group was significantly reduced compared with the empty vector (CONTROL) group (Figure 4C). Moreover, the expression levels of TTN-AS1 and miR-1271 in extracted tumor tissues were detected by RT-qPCR. The results showed that TTN-AS1 was downregulated in the TTN-AS1/shRNA group compared with the empty vector (CONTROL) group (Figure 4C). Conversely, miR-1271 was highly expressed in TTN-AS1/shRNA group compared with the empty vector (CONTROL) group (Figure 4D). The above results suggested that TTN-AS1 could promote tumor formation *in vivo*.

Discussion

Evidence has proved that lncRNAs play important roles in prostate cancer. For instance, lncRNA PCSEAT enhances cell proliferation of prostate cancer by activating EZH2, which may offer a potential therapeutic target[10]. LncRNA SNHG7 promotes cell proliferation and cycle progression in prostate cancer through miR-503/Cyclin D1 pathway[11]. The downregulation of lncRNA PVT1 inhibits the metastasis of prostate cancer cells by regulating p38[12]. LncRNA MALAT1 and HOTAIR have been reported to play opposite roles in estrogen-induced transcriptional regulation in prostate cancer cells[13].

LncRNA TTN antisense RNA 1 (TTN-AS1), located on 2q31.2, is a novel lncRNA that is reported to be upregulated in many cancers. For example, lncRNA TTN-AS1 is aberrantly expressed in hepatocellular carcinoma[14]. LncRNA TTN-AS1 functions as an oncogene in esophageal squamous cell carcinoma[15]. LncRNA TTN-AS1 enhances cell proliferation and aggressiveness in cervical cancer by targeting miR-573[16]. Our previous study showed that TTN-AS1 was upregulated in prostate cancer patients. Then, we conducted some functional experiments and found that after TTN-AS1 was knocked down, the proliferation and migration abilities of prostate cancer cells were inhibited, suggesting that TTN-AS1 could promote tumorigenesis of prostate cancer.

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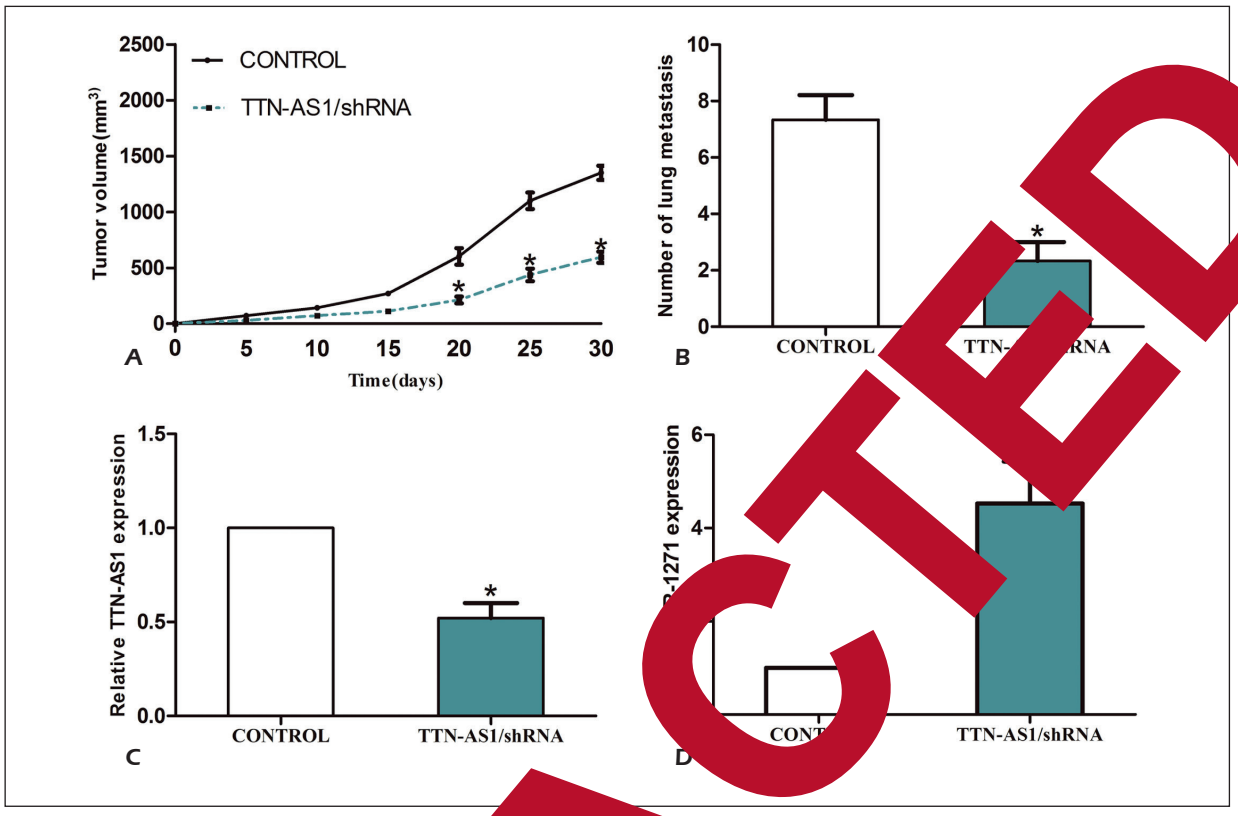

Figure 4. The knockdown of TTN-AS1 inhibited t… for… **A**, After tumor extraction, the tumor volume was calculated respectively in empty vector (CONTROL) o… -AS1… p and made into a graph. **B**, The number of metastatic nodules in the lung from the TTN-AS1/shRNA gro… …ificantly reduced compared to the empty vector (CONTROL) group. **C**, The relative expression of TTN-AS1 in tumors … …amined by RT-qPCR. **D**, The relative expression of miR-1271 in tumors were examined by RT-qPCR. … are presen… the mean ± SD of three independent experiments. *$p< 0.05$.

To further identify the …erlying …ism of how TTN-AS1 affects t… gr… tate cancer, miR-127… as s… out as the potential binding … RNA of … S1 by bioinformatic analy… experimenta… cation. MiR-1271 is … as a tumor suppressor in many car…mas, wh… ulates diverse biological p… ses. MiR-127… wnregulated in colorec… cancer and partici…es in regulating tum… velop… by targeting Wnt signaling pat…[17]. M… 271 co…d suppress cell metastasis … tic can… Recently, miR-1271 is …rted … ress… growth ability by target- …G in p… ncer[19].

… e prese… dy, miR-1271 expression was up… ed after the knockdown of TTN-AS1. M… -1271 could directly bind to TTN- …1, and miR-1271 expression was negatively …lated with TTN-AS1 in prostate cancer. A… results above suggested that TTN-AS1 migh… promote tumorigenesis of prostate cancer via sponging miR-1271. Besides, the tumorigenesis assay revealed that knockdown of TTN-AS1 could inhibit tumor formation and metastasis *in vivo*. Through the detection of TTN-AS1 and miR-1271 expressions in those extracted tumors, we found that TTN-AS1 was downregulated and miR-1271 was upregulated in nude mice administrated with TTN-AS1 shRNA.

Conclusions

The results of the study indicated that TTN-AS1 could enhance proliferation and migration abilities of prostate cancer cells by sponging miR-1271. These findings implied that lncRNA TTN-AS1 could act as a prospective therapeutic target for prostate cancer.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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