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Long non-coding RNA TTN-AS1 promotes cell proliferation and inhibits cell apoptosis in prostatic cancer by sponging miR-193a-5p

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Abstract. – OBJECTIVE: Prostatic cancer (PCa) is a common cancer in males. Long non-coding RNA (lncRNA) TTN-AS1 has been reported as an oncogene in diverse cancers. This study aimed to explore the functions and mechanism of TTN-AS1 in PCa.

MATERIALS AND METHODS: The levels of TTN-AS1 and miR-193a-5p in PCa cells (DU145, PC3, 22RV1, C4-2B, and LNCaP) were measured by qRT-PCR. The putative target of TTN-AS1 was predicted by starBase v2.0 online database, and this interaction was validated by Dual-Luciferase reporter assay. The cell viability and apoptosis rate in DU145 and PC3 cells were assessed by MTT assay and flow cytometry, respectively. The protein levels of CyclinD1, p21, p27, Bcl-2, Bax, and cleaved-caspase3 were detected by Western blot.

RESULTS: The relative expression of TTN-AS1 was apparently up-regulated, and the level of miR-193a-5p was strikingly down-regulated in PCa cells. The interaction between TTN-AS1 and miR-193a-5p was predicted by starBase v2.0 online database and verified by Dual-Luciferase reporter assay. The functional experiments indicated that TTN-ASI knockdown or miR-193a-5p inhibited cell viability and induced cell apoptosis rate in DU145 and PC3 cells. Furthermore, the recuperated experiments exhibited that miR-193a-5p inhibitor counteracted the inhibitory effect on cell viability and the promotion effect on cell apoptosis rate in DU145 and PC3 cells induced by TTN-AS1 silencing.

CONCLUSIONS: These data indicated that TTN-AS1 was dramatically up-regulated, and miR-193a-5p was significantly down-regulated in PCa cells. The functional and mechanistical experiments unraveled that lncRNA TTN-AS1 sponged miR-193a-5p to promote cell proliferation and repress cell apoptosis in prostatic cancer, and this new regulatory pathway may shed light on the mechanism of prostatic cancer.

Key Words.

LncRNA TTN-AS1, MiR-193a-5p, Cell proliferation, Cell apoptosis, Prostatic cancer.

Abbreviations

PCa = Prostatic cancer; FBS = fetal bovine serum; qRT-PCR = quantitative Real Time-Polymerase Chain Reaction; ANOVA = analysis of variance; EMT = epithelial-mesenchymal transition.

Introduction

Prostatic cancer (PCa) is a common malignant tumor of male reproductive system, and its morbidity ranks first among male tumors[1]. In recent years, the increasing incidence of prostate cancer in China is becoming a serious threat to men's life and health[2]. Due to the lack of evident symptoms in the early stage, PCa patients diagnosed in advanced stage often miss the best surgery opportunity[3,4]. However, the biological mechanism of PCa remains unclear. Long non-coding RNAs (lncRNAs) have been reported to involve in tumor development and progression and may be potential targets of tumor therapy[5-7]. LncRNAs play vital roles in tumor development and progression of PCa. Gu et al[8] reported that lncRNA HOXD-AS1 was highly expressed in castration-resistant prostate cancer cells and promoted PCa cell proliferation and chemotherapeutic resistance by recruiting WDR5. While another report[9] indicated that lncRNA XIST was apparently down-regulated PCa tissues and cells, and XIST modulated RKIP expression to inhibit cell proliferation and metastasis by sponging miR-23a. The above results implied that the dysregulation of lncRNAs affects the biological processes of PCa in different ways. However, the biological mechanism and functions of TTN-AS1 in PCa were still undefined. MicroRNAs (miRNAs), a class of small non-coding RNA with the length of 18-25 nt, have been documented to affect tumor progression in many tumors[10]. Many miRNAs were reported to have an aberrant expression

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in prostatic cancer. For example, miR-141[11], miR-1266, miR-185 and miR-30c-2[12], miR-30d-5p[13], and so on were apparently down-regulated in PCa tissues, cells, and some of them participated in the processes of tumor progression. Additionally, the dysregulation of miR-193a-5p has been documented in diverse cancer, including hepatocellular carcinoma[14], colorectal cancer[15], gastric cancer[16]. However, the mechanism of miR-193a-5p in PCa remains rarely reported. This study aimed to explore the functions and mechanism of TTN-AS1 in PCa. The results demonstrated that TTN-AS1 was dramatically up-regulated, and miR-193a-5p was significantly down-regulated in PCa cells. The further exploration implicated that lncRNA TTN-AS1 enhanced cell proliferation and blocked cell apoptosis in prostatic cancer by sponging miR-193a-5p, and this new regulatory pathway may provide novel target for PCa patients.

Materials and Methods

Cell Culture and Transfection

Five prostatic cancer cell lines DU145, PC3, 22RV1, C4-2B, LNCaP, and prostate epithelial cell lines RWPE-1 were purchased from the Chinese Academy of Medical Sciences (Beijing, China). Five prostatic cancer cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Sijichun, Hangzhou, China). Prostate epithelial cells RWPE-1 was cultured in KSFM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS). Small interference RNA (siRNA) targeting TTN-AS1 (si-TTN-AS1) and its mock (si-NC), TTN-AS1 overexpression vector (pcDNA-TTN-AS1) and its negative matched control (pcDNA), miR-193a-5p mimics (miR-193a-5p) and its scramble (miR-NC), miR-193a-5p inhibitor (anti-miR-193a-5p) and its matched control (anti-miR-NC) were purchased from GenePharma (Shanghai, China). The transfection was conducted using Lipofectamine™ 3000 according to the manual.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The RNA from cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Following detected the RNA concentration using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA), the RNA sample was reverse transcribed using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The quantitative PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China). The relative expression of TTN-AS1 and miR-193a-5p were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were obtained from Beijing Genomics Institute (BGI, Shenzhen, China) and list as follows: TTN-AS1: (Forward, 5'-CGGGAACAAGCCCTGTG-3', Reverse, 5'-CCGGCCCAAAGATGATG-3'); miR-193a-5p: (Forward, 5'-ACACTCCAGCTGGGTGGGTCTTTGCGGGCG-3', Reverse. 5'-TGGTGTCGTGGAGTCG-3'); GAPDH: (Forward, 5'-TGTTCGTCATGGGTGTGAAC-3', Reverse, 5'-ATGGCATGGACTGTGGTCAT-3') and U6: (Forward, 5'-CTCGCTTCGGCAGCACA-3', Reverse, 5'-AACGCTTCACGAATTTGCGT-3').

MTT Assay

DU145 and PC3 cells (5×10^3/well) were added into 96-well plate and cultivated in an incubator with the condition of 37°C, 5% CO_2 for 24 h, 48 h, 72 h. 20 μL of 5 μM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added into each well and incubated for another 4 h. Subsequently, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was injected into each well to dissolve the formazan. The absorbance at 490 nm was detected using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Apoptosis Assay

The cell apoptosis rate was performed using a FITC-Annexin V/Propidium iodide (PI) kit (Jingmei Biotechnology, Jiangsu, China). In brief, the transfected DU145 and PC3 cells were resuspended in phosphate-buffered saline (PBS) for three times. Then, 195 μL AnnexinV-FITC binding buffer and 5 μl AnnexinV-FITC were added into DU145 and PC3 cells (5×10^4-10^5) and incubated in the dark for 10 min. After adding 10 μL Propidium Iodide (PI), the sample was incubated for 10 min in the dark; then, the sample was detected using flow cytometry (BD Biosciences, San Jose, CA, USA).

Western Blot

The proteins from DU145 and PC3 cells were extracted using a protein extraction kit (Beyotime, Shanghai, China) and the protein concentration was measured using bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai,

China). 30 μg protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Subsequently, the membrane was blocked in non-fat milk for 2 h and then incubated with primary antibody overnight. The membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody. The chemiluminescence intensity was assessed using eyoECL Plus Kit (Beyotime, Shanghai, China). The primary antibody against CyclinD1, p21, p27 was purchased from Jingmei Biotechnology (Jiangsu, China), the primary antibody against Bcl-2, Bax, and cleaved-caspase-3 was bought from MXB Biotechnologies (Fuzhou, China) and the HRP-labelled secondary antibody was obtained from ZSGB-BIO (Beijing, China).

Dual-Luciferase Reporter Assay

The putative target of miR-193a-5p was predicted by starBase v2.0 online database (http://starbase.sysu.edu.cn). The sequence of TTN-AS1 or its mutant was cloned and then inserted into pGL3 vector (Promega, Madison, WI, USA), namely WT-TTN-AS1 and MUT-TTN-AS1, respectively. The co-transfection of luciferase reporter (WT-TTN-AS1 and MUT-TTN-AS1) and miR-193a-5p or miR-NC were conducted using Lipofectamine™ 3000 according to the manual. After transfection for 48 h, the Dual-Luciferase® Reporter Assay system (Promega, Madison, WI, USA) was used to assess the luciferase activity. The firefly luciferase activity was normalized by the *Renilla* luciferase activity.

Statistical Analysis

The data in this study was analyzed by SPSS.22.0 (IBM, Armonk, NY, USA). The comparison between two or more than groups were processed by Student's *t*-test or analysis of variance (ANOVA), respectively. Tukey's test was used to validate ANOVA for pairwise comparisons. Statistical significance was considered at $p<0.05$.

Results

TTN-AS1 Is Significantly Up-Regulated and MiR-193a-5p Is Strikingly Down-Regulated in PCa Cells

To explore the roles of TTN-AS1 and miR-193a-5p in PCa, the relative expression of TTN-AS1 and miR-193a-5p was measured by qRT-PCR. The results showed that TTN-AS1 was notably increased in prostatic cancer cells DU145, PC3, 22RV1, C4-2B, and LNCaP cells related to that in prostate epithelial cells RWPE-1 cells (Figure 1A). While the level of miR-193a-5p exhibited the opposite trends in PCa cells (Figure 1B). These results indicated that lncRNA TTN-AS1 was remarkably elevated and miR-193a-5p was conspicuously reduced in PCa cells.

TN-AS1 Knockdown Inhibits Cell Proliferation in DU145 and PC3 Cells

To investigate the functions of TTN-AS1 in PCa, the si-TTN-AS1 was transfected into DU145 and PC3 cells. The qRT-PCR results affirmed the knockdown efficiency, indicated by the down-regulation of TTN-AS1 in DU145 and PC3 cells (Figure 2A). Sub-

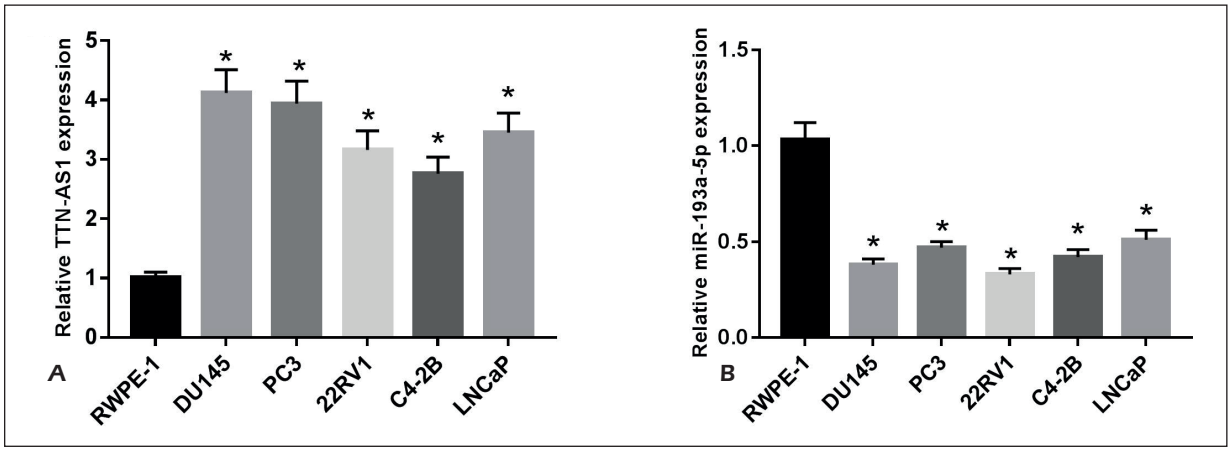

Figure 1. TTN-AS1 is significantly up-regulated and miR-193a-5p is strikingly down-regulated in PCa cells. The levels of TTN-AS1 (**A**) and miR-193a-5p (**B**) in prostatic cancer cells DU145, PC3, 22RV1, C4-2B, LNCaP cells, and prostate epithelial cells RWPE-1 cells were detected by qRT-PCR. *$p<0.05$.

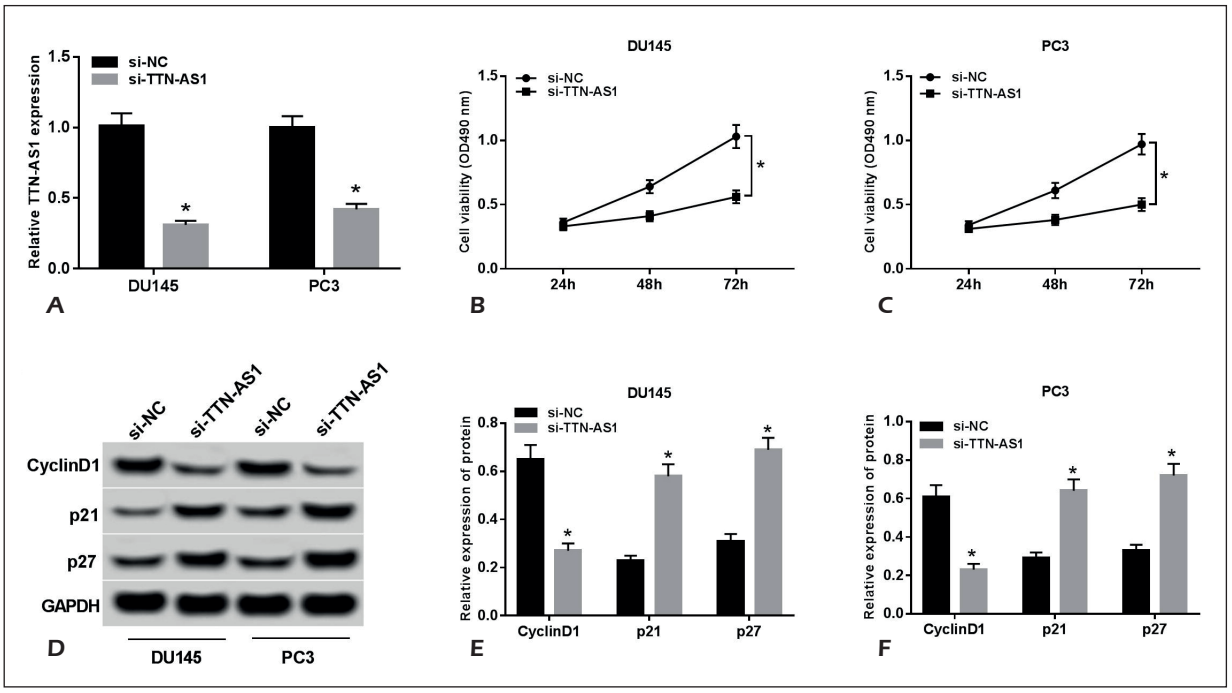

Figure 2. TTN-AS1 knockdown inhibits cell proliferation in DU145 and PC3 cells. The level of TTN-AS1 (**A**), cell viability (**B** and **C**), the protein levels of CyclinD1, p21 and p27 in si-TTN-AS1-transfected DU145 and PC3 cells (**D**-**F**) were measured by qRT-PCR, MTT assay, and Western blot, respectively. *$p<0.05$.

sequently, the MTT assay displayed that TTN-AS1 silencing constrained cell viability in DU145 and PC3 cells in contrast to that in si-NC group (Figure 2B and C). Since CyclinD1[17] was proliferation-related protein and p21[18], p27[19] were cell-cycle-arrest-related proteins, we further detect the protein levels of CyclinD1, p21, and p27 in si-TTN-AS1-transfected DU145 and PC3 cells. Western blot results exhibited that the protein level of CyclinD1 was decreased and the protein levels of p21, p27 were increased in DU145 and PC3 cells transfected with si-TTN-AS1 (Figure 2D-F). Taken together, these data implicated that TTN-AS1 knockdown suppressed cell proliferation in DU145 and PC3 cells.

TTN-AS1 Silencing Promotes Cell Apoptosis in DU145 and PC3 Cells

To further explore the functions of TTN-AS1 in PCa, cell apoptosis was further studied in DU145 and PC3 cells. The flow cytometry results showed that TTN-AS1 depletion enhanced apoptosis rate in DU145 and PC3 cells transfected with si-TTN-AS1 (Figure 3A-D). As Bcl-2 was apoptosis inhibitor protein, and Bax and cleaved-caspase3 were apoptosis-associated proteins[20], we further detected the protein levels of Bcl-2, Bax, and cleaved-caspase3 in si-TTN-AS1-transfected DU145 and PC3 cells. Western blot results indicated that the protein level of Bcl-2 was reduced in DU145 and PC3 cells with the transfection of si-TTN-AS1 compared with that in si-NC group; while the protein levels of Bax and cleaved-caspase3 exhibited the opposite trend (Figure 3E-G). To sum, the depletion of TTN-AS1 enhanced cell apoptosis in DU145 and PC3 cells.

MiR-193a-5p Overexpression Restrains Cell Proliferation and Enhances Cell Apoptosis in DU145 and PC3 Cells

To explore the functions of miR-193a-5p in PCa, miR-193a-5p mimics were transfected into DU145 and PC3 cells. The qRT-PCR confirmed the transfection efficiency, demonstrated by the apparently up-regulation of miR-193a-5p in DU145 and PC3 cells (Figure 4A). Furthermore, the MTT assay and flow cytometry results indicated that the overexpression of miR-193a-5p inhibited cell viability and promoted apoptosis rate in DU145 and PC3 cells transfected with miR-193a-5p mimics (Figure 4B and C). In addition, the Western blot results showed that the protein levels of CyclinD1, Bcl-2 were dramatically decreased, and the protein levels of p21, Bax were markedly increased in DU145 and PC3 cells

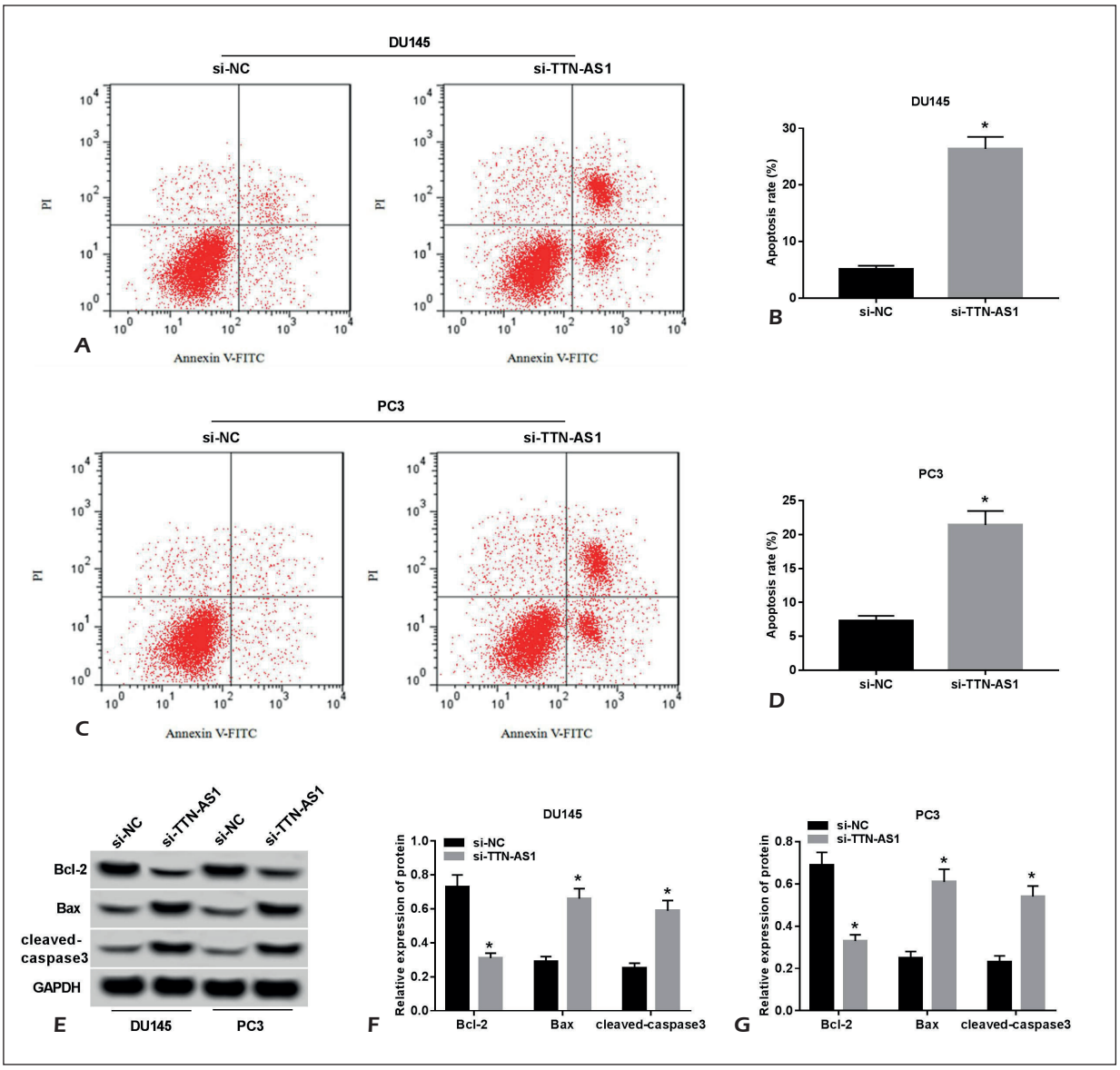

Figure 3. TTN-AS1 silencing promotes cell apoptosis in DU145 and PC3 cells. The cell apoptosis rate (**A-D**), the protein levels of Bcl-2, Bax, and cleaved-caspase3 (**E-G**) in si-TTN-AS1-transfected DU145 and PC3 cells were measured by flow cytometry and Western blot, respectively. *$p<0.05$.

transfected with miR-193a-5p mimics (Figure 4D-J). These data demonstrated that miR-193a-5p overexpression impeded cell proliferation and enhanced cell apoptosis in DU145 and PC3 cells.

MiR-193a-5p Is Negatively Interacted With TTN-AS1

To illustrate the biological mechanism of TTN-AS1 in PCa, the putative target of TTN-AS1 was predicted by starBase v2.0 online database. The results presented that miR-193a-5p had complementary sequences with TTN-AS1 (Figure 5A). Meanwhile, the qRT-PCR results showed that the level of miR-193a-5p was distinctly down-regulated in pcDNA-TTN-AS1-transfected DU145 and PC3 cells, while exhibited the opposite trend in si-TTN-AS1 group (Figure 5B). Following Dual-Luciferase reporter assay indicated that the transfection of miR-193a-5p strikingly down-regulated the luciferase activity of WT-TTN-AS1 reporter, while the luciferase activity of MUT-TNN-AS1 had no significant change in any group (Figure 5C and D). These results implied that miR-193a-5p was negatively interacted with TTN-AS1.

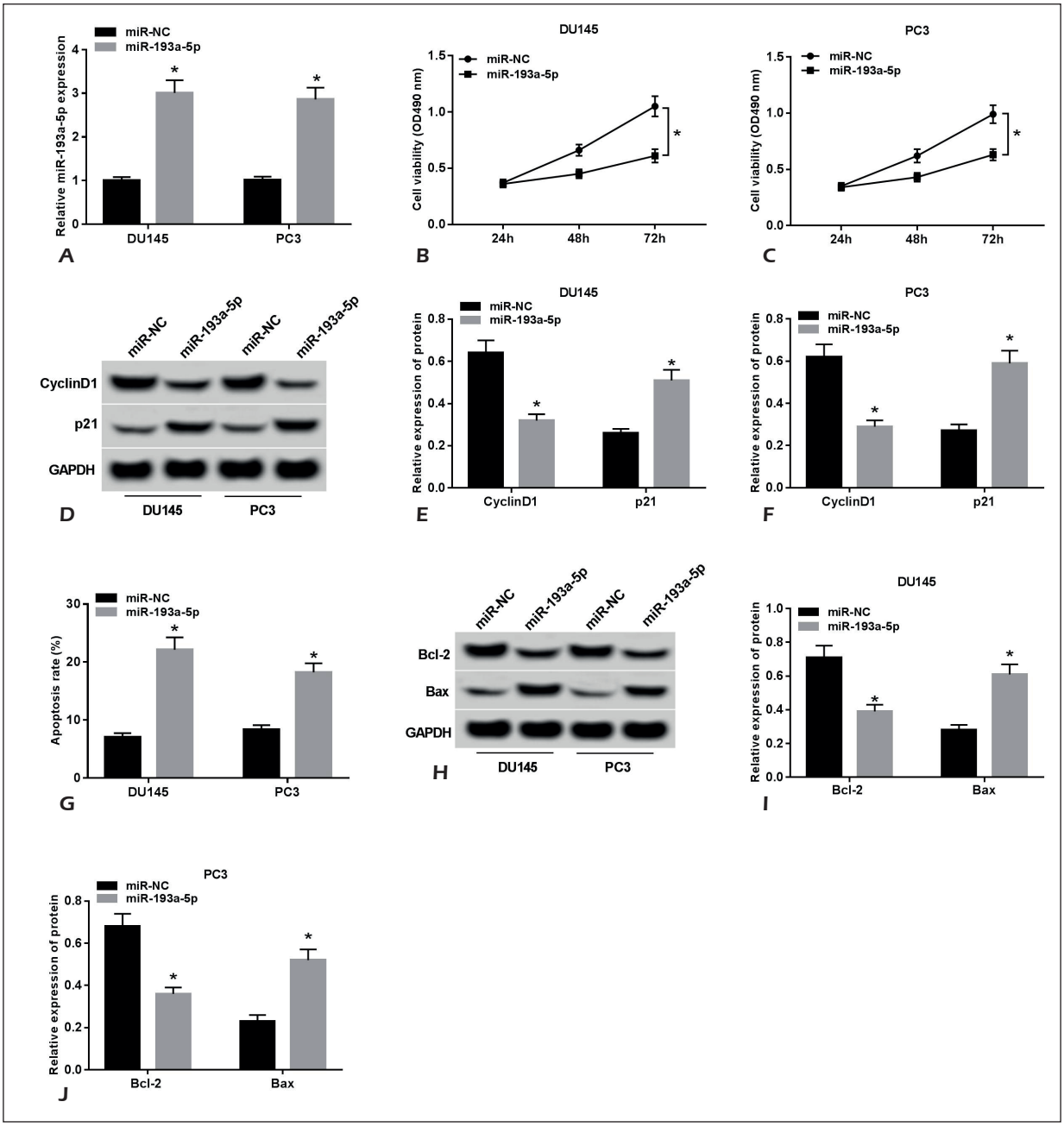

Figure 4. MiR-193a-5p overexpression restrains cell proliferation and enhances cell apoptosis in DU145 and PC3 cells. The level of miR-193a-5p (**A**), cell viability (**B** and **C**), apoptosis rate (**G**) and the protein levels of CyclinD1, p21 (**D**-**F**), Bcl-2 and Bax (**H**-**J**) in DU145 and PC3 cells transfected with miR-193a-5p were measured by qRT-PCR, MTT assay, flow cytometry, and Western blot, respectively. *$p<0.05$.

MiR-193a-5p Inhibitor Attenuates the Inhibitory Effect on Cell Proliferation and the Promotion Effect on Cell Apoptosis in DU145 and PC3 Cells Induced by si-TTN-AS1

To explore the functions of TTN-AS1 and miR-193a-5p in PCa, the restoration experiment was conducted. First, the qRT-PCR results exhibited that the level of miR-193a-5p was apparently increased in si-TTN-AS1-transfected DU145 and PC3 cells, while the level of miR-193a-5p partially reversed with the emergence of anti-miR-193a-5p (Figure 6A). Furthermore, the MTT assay indicated that anti-miR-193a-5p mitigated the suppressive effect on cell viability caused by si-TTN-AS1 in DU145 and PC3 cells (Figure 6B and

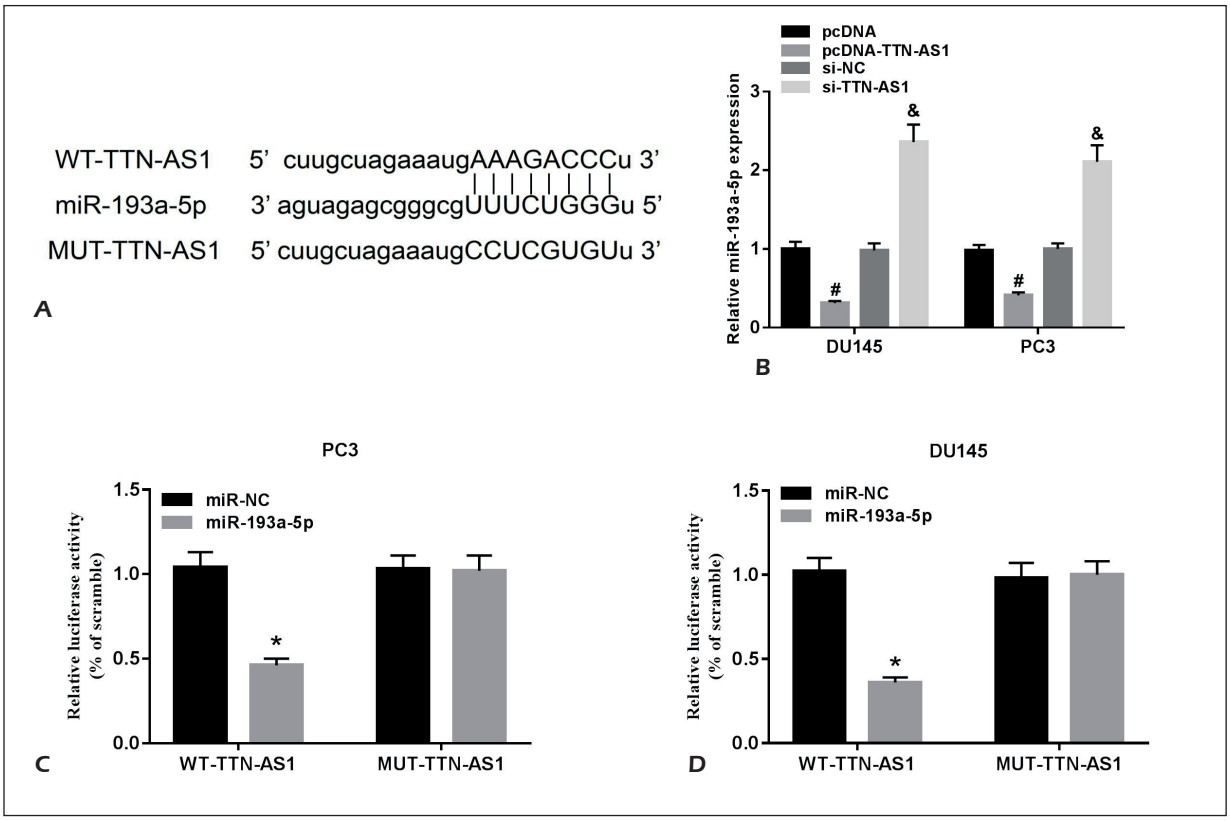

Figure 5. MiR-193a-5p is negatively interacted with TTN-AS1. **A**, Complementary binding sites between TTN-AS1 and miR-193a-5p. **B**, Level of miR-193a-5p in DU145 and PC3 cells transfected with pcDNA-TTN-AS1, si-TTN-AS1, or their matched controls were measured by qRT-PCR. The luciferase activity of WT-TTN-AS1 or MUT-TNN-AS1 reporter in PC3 (**C**) and du145 (**D**) cells transfected with miR-193a-5p or miR-NC was assessed by Dual-Luciferase reporter assay. *p<0.05.

C). However, the cell apoptosis rate showed the opposite trend compared with the results of cell viability (Figure 6H). In addition, the Western blot results exhibited that anti-miR-193a-5p alleviated the repressive effect on the protein levels of CyclinD1, Bcl-2, and the promotion effects on the protein levels of p21, Bax induced by si-TTN-AS1 in DU145 and PC3 cells (Figure 6D-G and I-L). Taken together, these data revealed that miR-193a-5p inhibitor counteracted the inhibitory effect on cell proliferation and the promotion effect on cell apoptosis in DU145 and PC3 cells induced by si-TTN-AS1.

Discussion

The pathogenesis of tumor is complicated, and the abnormal cell proliferation and apoptosis are one of the reasons for tumor development. Thus, inhibiting the abnormal cell proliferation and inducing the cell apoptosis in tumor are the approaches of tumor therapy[21-23]. LncRNA TTN-AS1 was reported to function as an oncogene in many cancers. This study focused on the mechanism and functions of TTN-AS1 in PCa. The results unraveled that lncRNA TTN-AS1 regulated tumor progression in prostatic cancer by sponging miR-193a-5p. Emerging evidence[24-26] indicated that lncRNA TTN-AS1 was strikingly up-regulated in lung adenocarcinoma, gastric cancer, esophageal squamous cell carcinoma, and so on, and its dysregulation is closely related to some physiological processes in tumor progression, such as cell proliferation, migration, and invasion. In this study, lncRNA TTN-AS1 was dramatically increased in PCa cells (DU145, PC3, 22RV1, C4-2B, and LNCaP). Further functional experiments indicated that TTN-AS1 silencing inhibited cell proliferation, and the down-regulation of proliferation-related protein CylinD1 and the up-regulation of proliferation inhibitor proteins p21, p27 further validated this result. The high apoptosis rate, the remarkably down-regulation of apoptosis inhibitor protein Bcl-

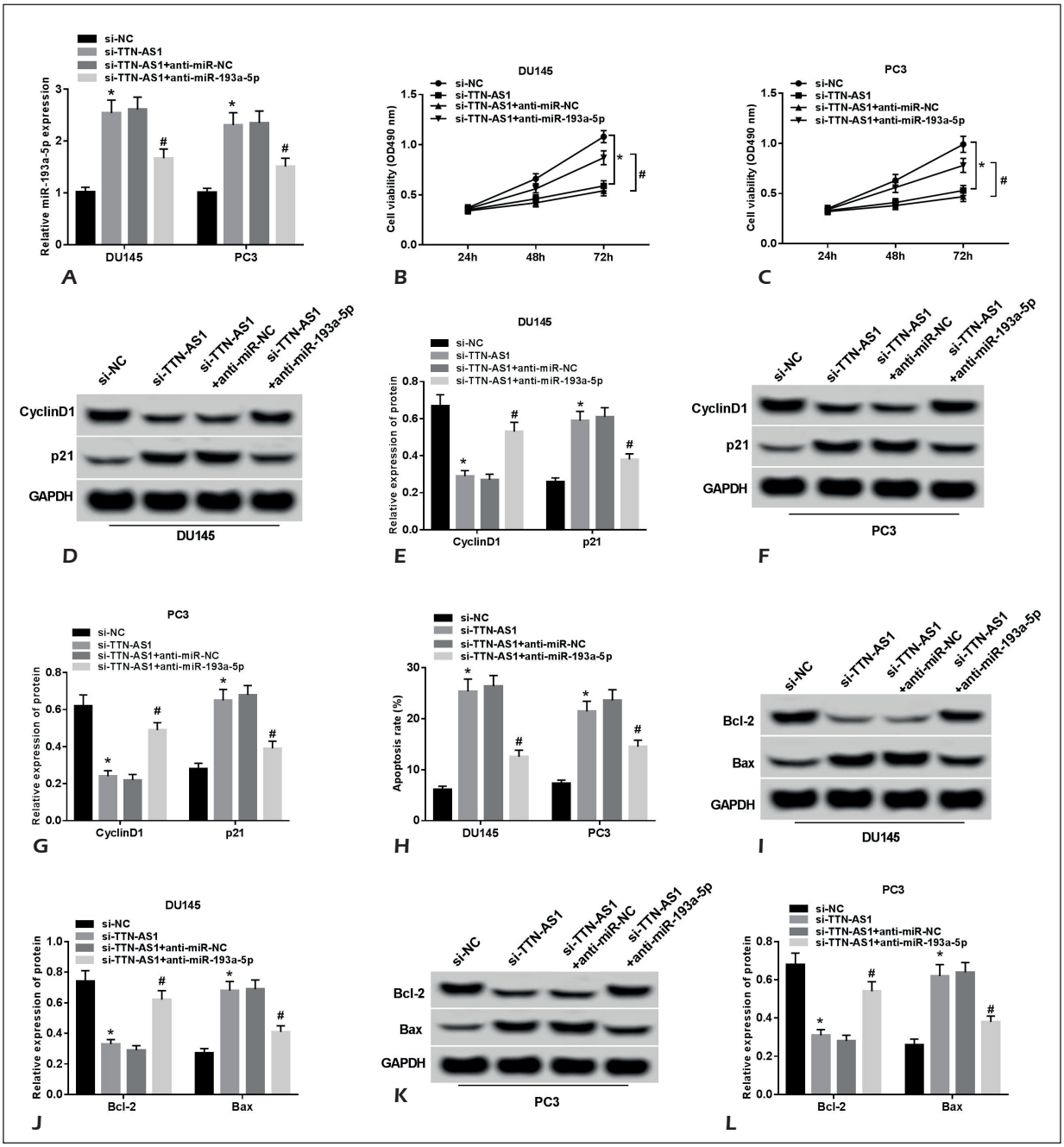

Figure 6. MiR-193a-5p inhibitor attenuates the inhibitory effect on cell proliferation and the promotion effect on cell apoptosis in DU145 and PC3 cells induced by si-TTN-AS1. The level of miR-193a-5p (**A**), cell viability (**B** and **C**), apoptosis rate (H) and the protein levels of CyclinD1, p21 (**D**-**G**), Bcl-2 and Bax (**I**-**L**) in DU145 and PC3 cells transfected with si-NC, si-TTN-AS1, si-TTN-AS1 or si-TTN-AS1+anti-miR-193a-5p were measured by MTT assay, flow cytometry and Western blot, respectively. *p<0.05.

2, and the significant up-regulation of apoptosis related protein Bax and cleaved-caspase3 implicated that TTN-AS1 depletion induced cell apoptosis in PCa cells. Accumulating evidence[27,28] indicated that lncRNA function as sponger to recruit miRNA and further decrease the miRNA expression. For example, a study in papillary thyroid cancer showed that lncRNA TTN-AS1 was strikingly elevated in papillary thyroid cancer tissues and cells, and TTN-AS1 modulated ZNRF2 expression by sponging miR-153-3p, contributing to the promotion effect on cell proliferation, migration, invasion,

and epithelial-mesenchymal transition (EMT)[29]. Another study in cervical cancer indicated that TTN-AS1 was apparently up-regulated in cervical cancer tissues and cells, and TTN-AS1 regulated cell proliferation and apoptosis by miR-573/E2F3 axis in cervical cancer[30]. In the present study, miR-193a-5p was markedly down-regulated in PCa cells. Subsequently, miR-193a-5p was predicted as a direct of TTN-AS1 by starBase v2.0 online database and validated by Dual-Luciferase reporter assay. The following functional and mechanistic experiments demonstrated that lncRNA TTN-AS1 function as a ceRNA to sponge miR-193a-5p, resulting in the promotion effect on cell proliferation and the inhibitory effect on cell apoptosis in PCa.

Conclusions

TTN-AS1 was drastically increased, and miR-193a-5p was distinctly reduced in PCa cells. TTN-AS1 enhanced cell proliferation and constrained cell apoptosis partially by down-regulating miR-193a-5p in PCa. This new regulatory pathway may provide a novel strategy for the treatment of PCa patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Yiwu Central Hospital. The methods used in this study were performed in accordance with relevant guidelines and regulations.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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