Iran. J. Biotechnol. January 2023;21(1): e 3405 DOI: 10.30498/ijb.2022.344921.3405

Research Article

Troponin T1 Promotes the Proliferation of Ovarian Cancer by Regulating Cell Cycle and Apoptosis

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Received: 2022/05/30; **Accepted:** 2022/11/16

Background: Troponin T1 (TNNT1) is implicated in human carcinogenesis. However, the role of TNNT1 in ovarian cancer (OC) remains unclear.

Objectives: To investigate the effect of TNNT1 on the progression of ovarian cancer.

Materials and Methods: The level of TNNT1 was evaluated in OC patients based on The Cancer Genome Atlas (TCGA). Knockdown or overexpression of TNNT1 using siRNA targeting TNNT1 or plasmid carrying TNNT1 was performed in the ovarian cancer SKOV3 cell, respectively. RT-qPCR was performed to detect mRNA expression. Western blotting was used to examine protein expression. Cell Counting Kit-8, colony formation, cell cycle, and transwell assays were performed to analyze the role of TNNT1 on the proliferation and migration of ovarian cancer. Besides, xenograft model was carried out to evaluate the *in vivo* effect of TNNT1 on OC progression.

Results: Based on available bioinformatics data in TCGA, we found that TNNT1 was overexpressed in ovarian cancer samples comparing to normal samples. Knocking down TNNT1 repressed the migration as well as the proliferation of SKOV3 cells, while overexpression of TNNT1 exhibited opposite effect. In addition, down-regulation of TNNT1 hampered the xenografted tumor growth of SKOV3 cells. Up-regulation of TNNT1 in SKOV3 cells induced the expression of Cyclin E1 and Cyclin D1, promoted cell cycle progression, and also suppressed the activity of Cas-3/Cas-7.

Conclusions: In conclusion, TNNT1 overexpression promotes SKOV3 cell growth and tumorigenesis by inhibiting cell apoptosis and accelerating cell-cycle progression. TNNT1 might be a potent biomarker for the treatment of ovarian cancer.

Keywords: Cell cycle, Cell apoptosis, Cell proliferation, Ovarian cancer Troponin T1

1. Background

Ovarian cancer (OC) is the foremost lethal and third most frequent tumor in female reproductive system (1). Among ovarian cancers, 92% of histological types are epithelial ovarian cancer, and two-thirds of reported cases are high-grade serous ovarian cancer (HGSOC) (2). The standard treatment for OC contains platinum-based surgical resection combined with chemotherapy, whereas the currently 5-year overall survival rate is roughly 47% due to relapse and chemoresistance (3). In addition, the difficulty of early detection, rapid distant metastasis, and rapid dissemination also contributes to the low overall survival of OC, despite that some prognostic biomarkers have been found (4). Therefore,

it is desirable to explore the underlying mechanisms of tumor progression and to reveal new clinical targets against the prognosis of OC patients.

The troponin complex is identified to be associated with actin filaments and functions as a vital part in regulating muscle relaxation and contraction (5). The troponin complex is formed by three subunits, including tropomyosin binding and thin filament anchoring subunit troponin T (TnT), calcium binding subunit troponin C (TnC) (6) and actomyosin ATPase inhibitory subunit troponin I (TnI), among which TnT plays the role of organizer (7). It is a protein (30–35 kDa) containing approximately 220–300 amino acids (7). Knockout of the TnT gene in mouse hearts results in embryonic lethality (8).

Troponin T1 (TNNT1), located on Chromosomal 19q13.4, contains 261 amino acids (5), and is associated with type 5 nemaline myopathy (9). Complete loss of TnT in Type I skeletal muscle caused by mutations in the TNNT1 gene results in severe nemaline myopathy and death in children (10). Recently, TNNT1 has been reported to contribute to the progression of colorectal cancer (11) and breast cancer (12). TNNT1 can also be used as a marker of cancer or disease, such as a prognostic indicator of colon adenocarcinoma (13) and gallbladder carcinoma (14), or as a new marker of retinal pigment epithelial cell immortalization (9) and rhabdomyosarcoma (RMS) (15). TNNT2 and TNNT3 can also serve as biomarkers for cancer progression (16, 17). Our previous research identified that TNNT1 expression was elevated in ovarian cancer tissue samples using the Cancer Genome Atlas (TCGA) data. However, whether the up-regulation of TNNT1 was related to the occurrence and development of ovarian cancer remained unclear.

2. Objectives

This study aims to investigate the function of TNNT1 during the progression of ovarian cancer.

3. Materials and Methods

3.1. Data Sources

Data from ovarian cancer and normal tissue samples were collected from The Cancer Genome Atlas portal (TCGA; www.cancergenome.nih.gov). This dataset contained a total of 514 ovarian samples, including 426 tumor samples and 88 adjacent normal samples. The expression of TNNT1 in these samples was analyzed.

3.2. Cell Culture and Cell Transfection

Human ovarian cancer cell SKOV3 and normal ovarian epithelial cell line IOSE80 were obtained from the Cell center of Peking Union Medical. The cells were cultured in the complete culture medium which consists of Roswell Park Memorial Institute-1640 complete medium (RPMI-1640; Invitrogen), 100 $U.mL^{-1}$ penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; Gibco). The cells were kept in a humidified incubator at the constant temperature of 37 °C supplied with 5% CO_2.

SKOV3 cells were transfected with two small interfering RNA targeting TNNT1 (siTNNT1#1, siTNNT1#2) or overexpression plasmids of TNNT1 (pcDNA3.1/TNNT1) to establish TNNT1 knockdown or TNNT1 overexpression model. These siRNAs, plasmids, and controls were synthesized or provided by Hippobio (Huzhou, China). The sequences were respectively shown as: siTNNT1#1: 5'-CUCUGGACAUUGACUACAUTT-3',siTNNT1#2: 5'-GCGUUGACUUCGAUGACAUTT-3', siCtrl:5'-UUCUCCGAACGUGUCACGU-3'. Lipofectamine 2000 (Thermo Fisher Scientific) was applied for cell transfection.

TNNT1 shRNA (shTNNT1) and control shRNA (shCtrl) were purchased from Hippobio (Huzhou, China). The 293T cells were co-transfected with shRNA and lentiviral packaging vectors. The supernatant of virus-containing medium was collected by centrifugation after transfection for 72 hours. 10 $\mu g.mL^{-1}$ of polybrene was added to SKOV3 cells to enhance lentivirus infection.

3.3. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

RNA from transfected siRNA and plasmid or non-transfected SKOV3 cells was extracted by TRIzol Reagent (Invitrogen). The quality and quantity of RNA was determined via Nano.Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA was then prepared and reverse-transcribed into cDNA using TransScript® Two-Step RT-PCR SuperMix (TransGen Biotech). RT-qPCR was carried out using the ABI 7500 real-time PCR system (Applied Biosystems) with the SYBR Premix Ex Taq II kit (Takara). GAPDH was used as the internal reference.

The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the relative expression of TNNT1 (18). The sequence of RT-qPCR primers was as follows:

TNNT1,
Forward: 5'-TGATCCCGCCAAAGATCCC -3',
Reverse: 5'-TCTTCCGCTGCTCGAAATGTA-3';
Cyclin D1,
Forward: 5'- GCTGCGAAGTGGAAACCATC-3',
Reverse: 5'- CCTCCTTCTGCACACATTTGAA-3';
Cyclin E1,
Forward: 5'- AAGGAGCGGGACACCATGA-3',
Reverse: 5'-ACGGTCACGTTTGCCTTCC-3';
GAPDH,
Forward: 5'-TGTGGGCATCAATGGATTTGG-3',
Reverse: 5'-ACACCATGTATTCCGGGTCAAT-3'.

3.4. Western Blot

Transfected SKOV3 cells were primarily lysed by radioimmunoprecipitation assay (RIPA) lysis buffer for protein extraction, and the concentration was detected by bicinchoninic acid assay (BCA). Protein samples were separated through 10% SDS-PAGE, followed by immunoblotting onto polyvinylidene difluoride (PVDF) membranes. Before adding TNNT1 (1:1500 dilution, Sigma) or GAPDH (1:2000) primary antibody for incubation at 4 °C overnight, the membranes containing sample bands were firstly blocked with 5% fat-free milk. After washing the membranes by PBS for three times, horseradish peroxidase (HRP) coupled secondary antibody (1:10000 dilution) was used to incubate the membrane. Enhanced chemiluminescence (ECL) reagent (Beyotime, China) and ImageJ software were used for visualization analysis of protein bands.

3.5. Cell Viability

Cell viability was examined through Cell Counting Kit-8 (CCK-8, Beyotime). The siRNAs or plasmids transfected SKOV3 cells were plated into a 96-well plate with the concentration at 5×10^3 cells/well. SKOV3 cells were adherent to the wall for 24, 48, 72 and 96 hours, separately. After that, the cell supernatant was washed with PBS, and 10 µL of CCK-8 in 90 µL of RPMI-1640 medium was added successively. After incubation for 2 hours, the absorbance was measured at 450 nm through a microplate reader (Bio-Rad Laboratory).

3.6. Colony Formation Assay

A total of 1×10^3 cells were plated in triplicate in each well of the 6-well plates, supplied with 3 mL complete culture medium. Fresh medium was replaced every 72 hours. After 14 days, the colonies were fixed by methanol for 30 minutes and stained by 0.1% crystal violet for 30 minutes. Lastly, the colonies were washed by clean water and dried in room temperature. The images of the colonies

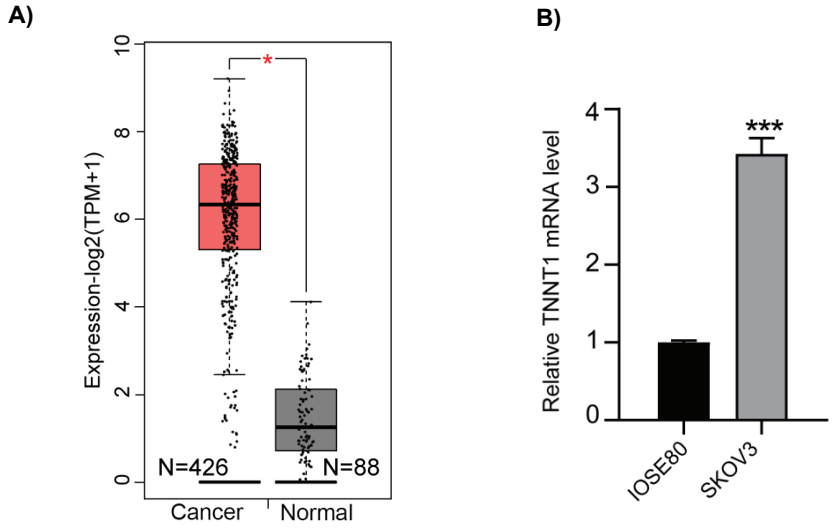

Figure 1. TNNT1 is strongly expressed in ovarian cancer. A) The comparison of TNNT1 expressing level in cancer samples (n=426) and normal samples (n=88) from TCGA data. **B)** The mRNA level of TNNT1 in normal cell line IOSE80 and ovarian cancer cell line SKOV3. *P < 0.05, ***P < 0.001. TNNT1, Troponin T1; TCGA, The Cancer Genome Atlas.

were captured by a camera and counted under the microscope. Colonies were defined when there were more than 50 cells in each cell cluster.

3.7. Transwell Assay

For transwell analysis, 1×10^5 transfected cells at the volume of 200 uL were added to the upper chamber coated with 200 $g.L^{-1}$ Matrigel. The lower chamber was fulfilled with 500 µL complete culture medium. The cells were reserved at a constant temperature at 37 °C with 5% CO_2 for 24 hours before the staining procedure with 0.1% crystal violet. With the staining procedure completed, the transferred cells were observed through an inverted microscope and then counted.; NINGBO SUNNY INSTRUMENTS

3.8. Xenograft Model

10-week-old athymic mice (Rj: ATHYM-foxn1nu/nu) weighting 20-22 g were used as experimental materials. A group of 10 female nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and randomly divided into shCtrl and shTNNT1 group. All animal experiments followed the guidelines provided by the Local Ethics Committee of Shandong First Medical University. The 1×10^6 transfected SKOV-3 cells were suspended in 50 µL PBS and implanted subcutaneously to establish the xenograft tumor model. When the tumor volume was approximately up to 160 mm^3 and lasted for about 10 days, the tumor was collected (the tumor volume was calculated as volume = 0.5 ×width ^{2}×length, by a caliper) (19).

3.9. Cell Cycle

The transfected SKOV-3 cells were enzymatically digested and collected, and 1×10^6 cells were suspended in PBS and fixed overnight in precooled ethanol at 4 °C. After treated with RNaseA (Sigma, USA), they were stained with propidium iodide (PI) staining solution (Sigma, USA). Cell cycle was detected by flow cytometry (FACSCanto TM II, BD Biosciences) (20).

3.10. Caspase-3/caspase-7 Activity Measurement

On the multifunctional enzyme labeling instrument (Biotek Synergy), Apo-ONE ® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI #G7791) was used to measure Caspase 3/7 activity. The excitation wavelength was 498nm, and the emission wavelength was 521nm. The experiments were repeated in quadruplicate (21).

3.11. Statistical Analysis

Data were expressed as mean ± SD, and analyzed using SPSS 22.0 software (IBM, USA). The differences between groups were conducted by Student's *t* -test. $p < 0.05$ was considered statistically significant.

4. Results

4.1. TNNT1 is Up-R egulated in Ovarian Cancer

Firstly, the expression of TNNT1 in ovarian cancer tissue samples and corresponding control tissues was analyzed. The results showed that TNNT1 was highly expressed in ovarian cancer samples compared with normal samples (**Fig. 1A**). The mRNA level of TNNT1 in ovarian cancer cell line SKOV3 was significantly increased, comparing to IOSE80 cells (**Fig. 1B**). These data implied that TNNT1 might function as an oncogenic factor during the OC progression.

4.2. Knockdown of TNNT1 Suppresses the Cell Proliferation and Migration

The impact of TNNT1 on OC cell proliferation was studied by "functional loss" strategy. Western blot and RT-qPCR experiments together proved that TNNT1 was decreased in SKOV3 cells transfected with siTNNT1#1 and siTNNT1#2 (**Fig. 2A, 2B**). Compared with Ctrl group, SKOV3 cell viability was significantly inhibited after TNNT1 knockdown (**Fig. 2C**). Consistently, the number of SKOV3 cells cloned or migrated after TNNT1 knockdown was significantly reduced as demonstrated by transwell assay and cell colony formation assay (**Fig. 2D, 2E**). The results above showed that TNNT1 knockdown inhibited the growth and migration of OC cells.

4.3. Overexpression of TNNT1 Promoted the Cell Proliferation and Migration

To further reveal the function of TNNT1 on ovarian cancer cell amplification, the "Gain of function" strategy was used. The overexpression efficiency of TNNT1 was verified by RT-qPCR and Western blot (**Fig. 3A, 3B**). The viability of SKOV3 cells was increased after transfection with PCDNA-TNNT1 plasmid (**Fig. 3C**). In addition, TNNT1 overexpression promoted the clonal formation ability and cell migration ability of SKOV3 cells (**Fig. 3D, 3E**). These results illustrated

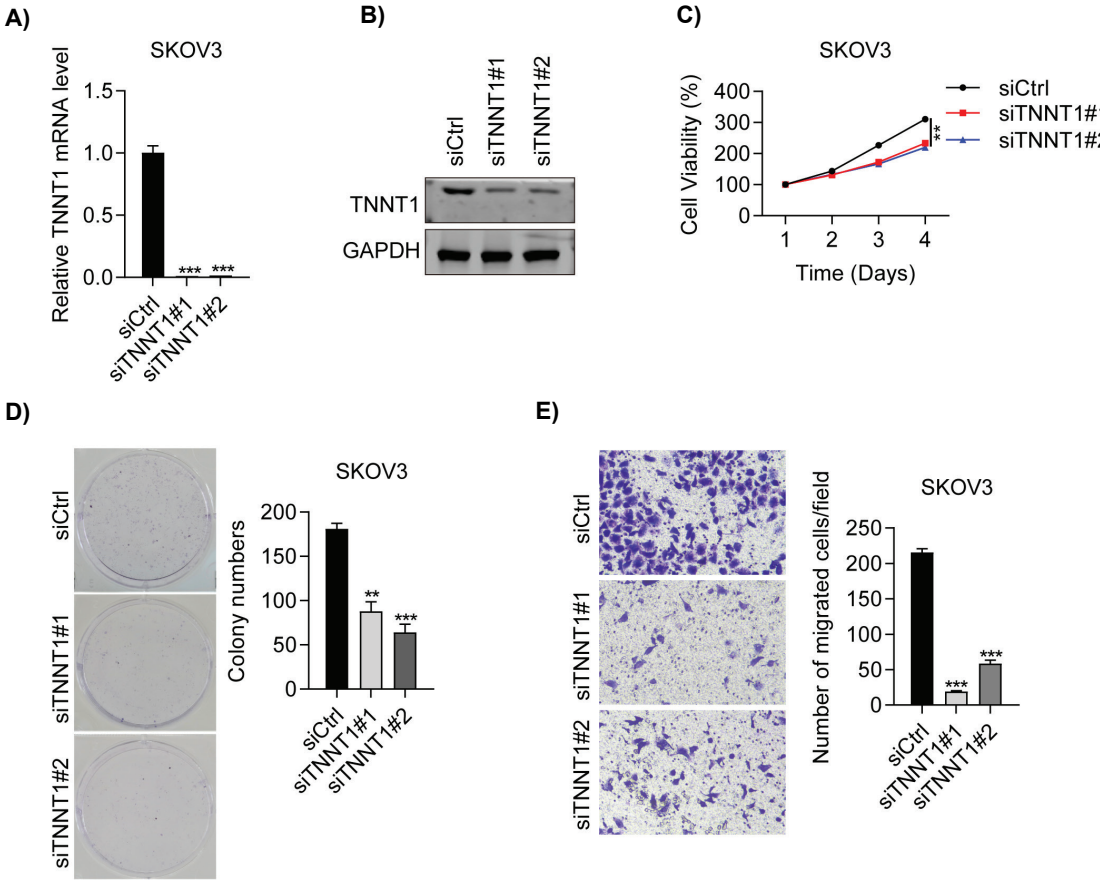

Figure 2. TNNT1 knockdown hinders cell proliferation, colony formation and migration. A) Knockdown of TNNT1 in SKOV3 cells was tested by RT-qPCR. **B)** Knockdown of TNT1 in SKOV3 cells was examined by Western blot. **C)** CCK-8 results of cell viability in SKOV3 cells after TNNT1 knockdown. **D)** Colony formation results of SKOV3 cells after TNNT1 knockdown. **E)** Transwell results of cell migration in SKOV3 cells after TNNT1 knockdown. **P < 0.01, ***P < 0.001. RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; CCK-8, Cell Counting Kit-8.

that TNNT1 overexpression significantly promoted the growth and proliferation of SKOV3 cells.

4.4. Knockdown of TNNT1 Retarded Tumor Growth in vivo

A Xenograft model was constructed to validate the oncogenic roles of TNNT1 in OC. The down-regulation of TNNT1 significantly hindered tumor growth of OC, which was consistent with us *in vitro* results (**Fig. 4A**). Meanwhile, the tumor volume of shTNNT1 group was significantly smaller than shCtrl group (**Fig. 4B**). These results demonstrated that TNNT1 silencing in SKOV3 xenograft models can delay tumor growth.

4.5. TNNT1 Regulated Cell Cycle and Cell Apoptosis

TNNT1 overexpression significantly promoted cell cycle progression by reducing the number of G0/G1 phase cells, compared with Ctrl group (**Fig. 5A**). Both Cyclin D1 and E1 were key factors in initiating cell cycle and promoting the progression from G1 phase to S phase (20). The expression of Cyclin D1 and E1 was significantly increased after transfection of SKOV3 cells with pcDNA-TNNT1 plasmid (**Fig. 5B**). Activation of Caspase 3/caspase 7 was regarded as a decisive molecular marker of apoptotic cell death (22). TNNT1 overexpression decreased the activity of Caspase 3/caspase 7 (**Fig. 5C**). These results suggested that TNNT1 could regulate cell cycle and apoptosis.

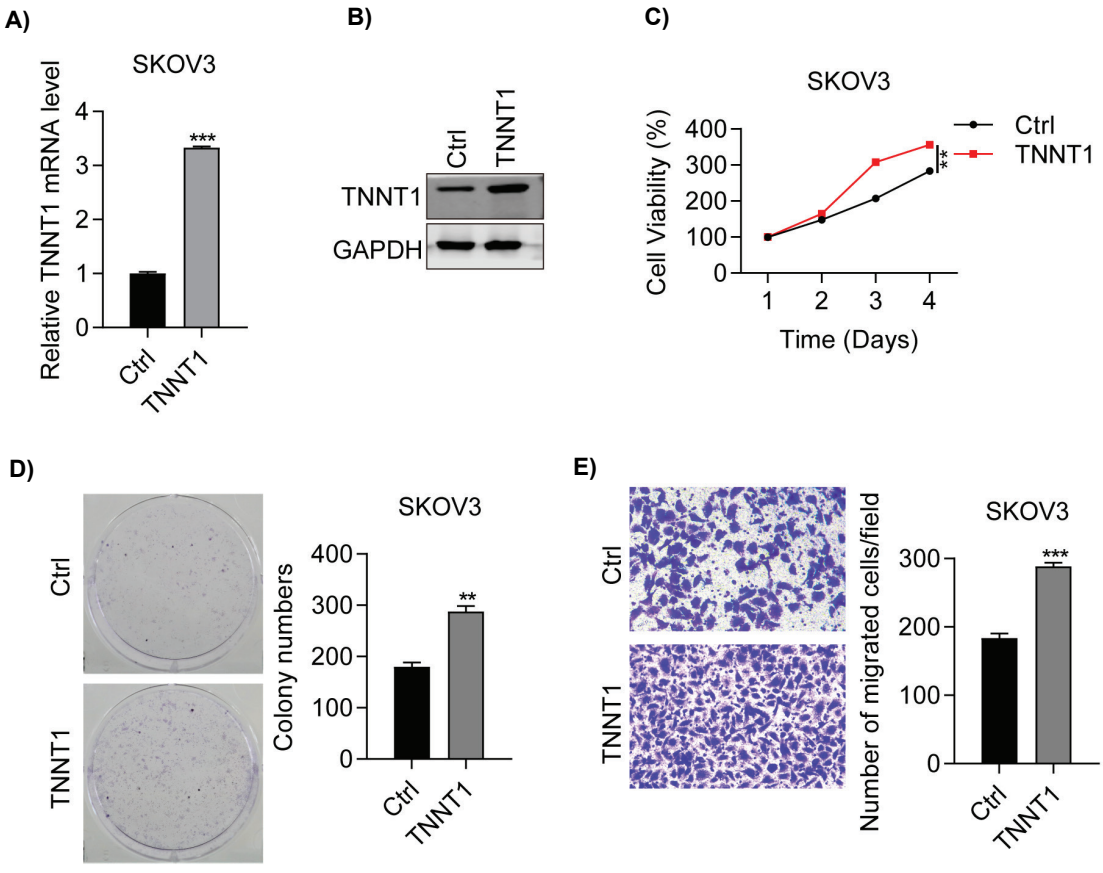

Figure 3. Overexpression of TNNT1 promoted colony formation, cell migration and cell proliferation. A) Overexpression of TNNT1 was examined by RT-qPCR in SKOV3 cells. **B)** Overexpression of TNNT1 was examined by Western blot in SKOV3 cells. **C)** CCK-8 results of cell viability in SKOV3 cells following TNNT1 overexpression. **D)** Colony formation results of SKOV3 cells following TNNT1 overexpression. **E)** Transwell results of cell migration in SKOV3 cells following TNNT1 overexpression. **P < 0.01, ***P < 0.001.

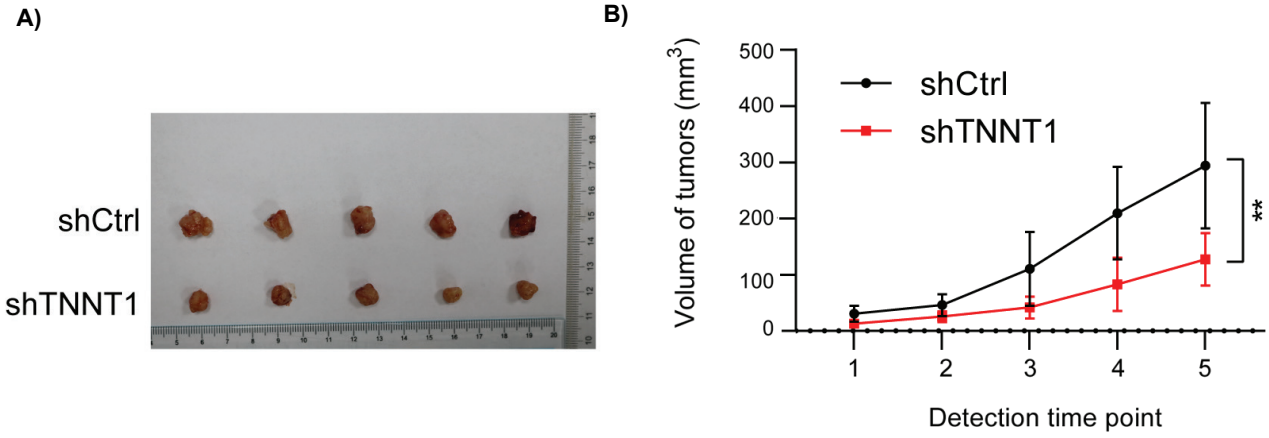

Figure 4. **Inhibition of tumor growth in tumor-bearing mice model by knocking down TNNT1. A)** Tumor size of nude mice between shCtrl group and shTNNT1 group. **B)** Comparison of the volume of ovarian tumor between shTNNT1 group and shCtrl group. **P < 0.01.

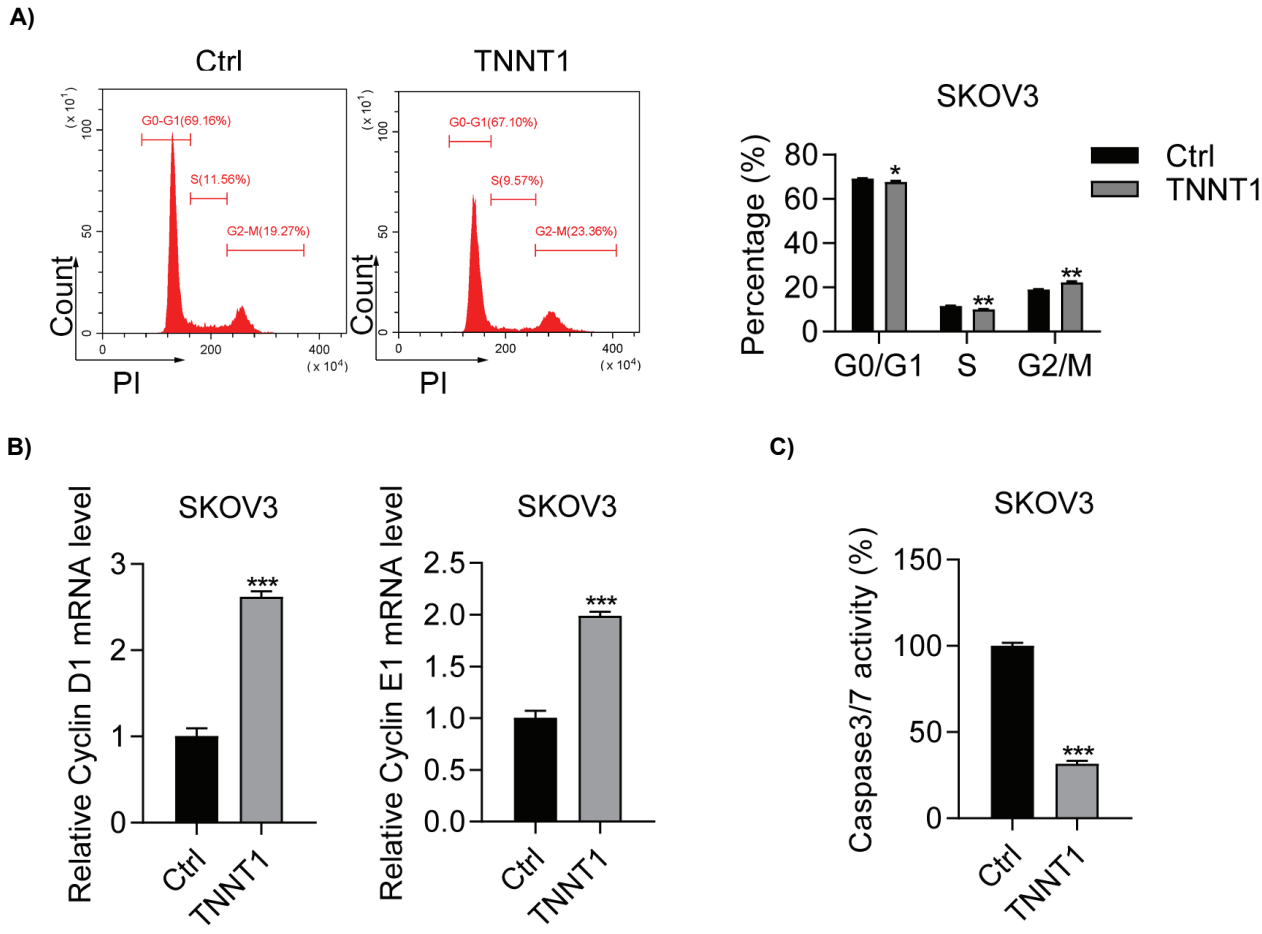

Figure 5. TNNT1 regulated cell cycle and apoptosis. A) Distribution of SKOV3 cell cycle following TNNT1 overexpression was measured by flow cytometry analysis. **B)** The relative expression level of Cyclin D1 and Cyclin E1 in SKOV3 cells were measured by RT-qPCR following TNNT1 overexpression. **C)** The activity of Caspase 3/caspase 7 was measured in SKOV3 cells following TNNT1 overexpression. *P < 0.05, **P < 0.01, ***P < 0.001.

5. Discussion

Ovarian cancer is one type of gynecological malignancies with the most lethal characteristic in females and a poor 5-year survival rate (1, 23). It is essential to reveal the mechanism of OC hallmarks for developing novel combination therapy strategies. Troponin T (TnT) is essential for regulating striated muscles contraction, which possess a vital function in the ability of calcium to regulate actin thin filament (5). Many reports have showed that complete loss of TNT in slow skeletal muscle due to mutations in the TNNT1 gene can lead to severe nemaline myopathy (24). Interestingly, in recent years, accumulated studies have reported the tumor-related functions of TNNT1. However, the exact role of TNNT1 in ovarian cancer remains to be investigated. In this study, we showed that the growth of ovarian tumor cell line SKOV3 was inhibited after TNNT1 knockdown. Knockdown of TNNT1 also inhibited tumor growth *in vivo*. Induction of endogenous TNNT1 level in SKOV3 cells significantly promoted SKOV3 cell proliferation. Several studies have demonstrated that TNNT1 was involved in growth and differentiation of cancer cells. Shi *et al* proved that TNNT1 was greatly elevated in breast cancer sections and facilitated cell proliferation by promoting G1/S transition (12). Chen *et al* reported that TNNT1 was upregulated in colorectal cancer sample tissues and cell lines, and overexpression of TNNT1 promoted cell progression

of CRC (11). In this paper, we revealed that TNNT1 promoted tumor growth and proliferation of ovarian cancer cells. In addition, TNNT1 also induced the migratory capacity of ovarian cancer cells, implicating that TNNT1 overexpression in ovarian cancer may facilitate metastasis.

A fundamental aspect of cancer cells was abnormal cell cycle regulation (25). G1/S transition is critical for cell proliferation and its dysfunction may lead to tumorigenesis (26). The main genes involved in cell cycle regulation were Cyclins and CDK proteins (27). Cyclin D1 and Cyclin E are central factors that initiate cell cycle and promote transition of cell cycle G1/S (20). Our study found that TNNT1 overexpression reduced the ratio of G0/G1 phase by promoting the expression of Cyclin D1 and Cyclin E1. Apoptosis is precisely regulated in normal cells, and dysregulation of apoptosis also exists in tumor cells (28). Due to the apoptosis stages, Caspases activity was used as a apoptosis-specific target to directly observe apoptosis (29). The ratio of Caspase-3 (CASP3) to caspase-7 (CASP7) has been extensively used as the cell apoptosis index (30). We demonstrated that TNNT1 overexpression down-regulated the activation of Caspase 3/caspase 7, suggesting that TNNT1 inhibited cell apoptosis. These results suggested that TNNT1 played a tumor promoting role in ovarian cancer by inducing cell-cycle progression and inhibiting apoptosis.

6. Conclusion

In conclusion, we reported for the first time regarding the function of TNNT1 in OC progression. The downregulation of TNNT1 reduced the viability, colony formation, migration, and tumorigenesis of OC cells. TNNT1 overexpression promoted cell cycle progression by improving cyclin level and inhibited apoptosis by decreasing the ratio of Caspase 3/caspase 7. These results implied that TNNT1 might be as a promising biomarker for OC therapy. The mechanism of TNNT1 regulating the occurrence and worsening of ovarian cancer needs to be further studied.

Acknowledgements

Not applicable.

Conflict of interest

The authors declare no potential conflict of interest

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