Original Article

Knockdown of tankyrase 1 inhibits the progression of gastric adenocarcinoma via regulating human telomerase reverse transcriptase and telomeric repeat binding factor 1

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Background: Gastric cancer is one of the most lethal cancers. Aberrant expression levels of genes are frequently associated with cell immortalization and the occurrence of tumors. In this study, we aimed to investigate the role of tankyrase 1 (*TANK1*) in gastric adenocarcinoma and clarify the underlying mechanism.

Methods: The messenger RNA (mRNA) levels of *TANK1*, human telomerase reverse transcriptase (*h-TERT*), and telomeric repeat binding factor 1 (*TRF1*) in clinical specimens and SGC-7901 cells were measured via real-time quantitative polymerase chain reaction (RT-qPCR). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and immunohistochemistry (IHC) assays were utilized to observe the cell apoptosis as well as Ki67 and *h-TERT* expression in tumor-bearing models. The effects of *TANK1* antisense oligonucleotides (TANK1 ASODN) on viability and apoptosis of SGC 7901 cells were evaluated by cell counting kit-8 and flow cytometry analysis.

Results: We found that *TANK1* and *h-TERT* were both increased in gastric adenocarcinoma, while *TRF1* was decreased. Tumor-bearing models demonstrated that TANK1 ASODN appeared to be effective in inhibiting tumor growth and decreasing the expression of *h-TERT*. Additionally, TANK1 ASODN inhibited the viability and promoted apoptosis of SGC-7901 cells. Moreover, the mRNA levels of *h-TERT* and *TRF1* were modulated by TANK1 ASODN.

Conclusions: This study revealed that TANK1 ASODN inhibits the proliferation and induced the apoptosis of gastric adenocarcinoma cells via manipulating the expression levels of *h-TERT* and *TRF1*.

Keywords: Tankyrase 1 (TANK1); human telomerase reverse transcriptase (h-TERT); telomeric repeat binding factor 1 (TRF1); gastric adenocarcinoma

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Introduction

Gastric cancer is a leading cause of mortality from digestive tract cancer, which is particularly prevalent in Asian populations (1,2). The incidence of gastric cancer has increased annually worldwide. Following the development of the electronic gastroscope, the detection rate of early gastric cancer has greatly increased, but its pathogenesis is still obscure.

Telomere and telomerase are hot topics in basic tumor research in recent years. A telomere is a specific DNA-protein complex in eukaryotic linear chromosomes, which contributes to the stability of chromosome structure and functions. Telomerase is an RNA-containing reverse

transcriptase composed of protein and RNA in the vast majority of tumors (3), which is crucial for the survival of cancer cells. Given that the activation of telomerase and the maintenance of telomere length are the bases of cell immortalization and unlimited proliferation (4), they have become new tumor markers and anti-cancer targets. Human telomerase reverse transcriptase (h-TERT) is the main component of human telomerase, and recurrent mutations in the promoter of *h-TERT* (5,6) is closely associated with telomerase activity. Telomeric repeat binding factor 1 (*TRF1*) is a telomeres double-stranded DNA binding protein in mammals, which has negative regulation effects on telomere length (7,8). A previous study has shown that tankyrase (TANK), a member of the poly (ADP-ribose) polymerase (PARP) superfamily, is identified to bind with the telomeric protein *TRF1* and regulate telomere function (9). It is known that TANK induces ADP ribosylation of *TRF1* and inhibits its binding to telomeres, suggesting that TANK is a positive regulator of telomere length and may be a target of tumor gene therapy. Recently, researchers have confirmed that TANK1 is highly expressed in neuroblastoma and bladder cancer (10,11).

Telomere, telomerase, and telomere binding proteins partaking in the regulation of DNA damage repair, cell cycle, mitosis, and cell apoptosis, are in close association with aging and tumors. It has been reported that the ablation of *h-TERT* induces cellular senescence and inhibits the growth of gastric cancer cells (12). Down-regulation of *TRF1* and *TRF2* is important for the maintenance of telomeric DNA (13). In addition, co-inhibition of *TANK1* and telomerase activity exerts a synergistic effect on telomere length shortening in gastric cancer cells (14). A considerable body of evidence indicates that human telomerase antisense oligonucleotides (ASON) coupled with oligoadenylic acid has significant inhibitory effects on the growth of prostate cancer, glioma and liver cancer (15-17). These findings suggest that inhibition of telomerase activity by ASON technology can inhibit tumor cell growth, indicating that if TANK1 is inhibited by ASON, tumor cell growth may also be suppressed. Ji et al constructed antisense human TANK1 RNA retroviral vector and revealed its inhibition on tongue cancer cells (18). A previous study has reported that TANK1 was up-regulated in osteosarcoma cells and TANK1-ASODN could suppress the proliferation, migration and invasion through Hippo/YAP pathway in human osteosarcoma cells (19). It was previously identified that *TANK1* is significantly upregulated in gastric cancer tissues (20); however, another study presented that TANK is not increased in gastric cancer (13). These controversial results caught our attention. Thus, the purpose of this study was to investigate the expression of *TANK1* in cancer and adjacent cancer tissues in gastric adenocarcinoma patients. The effects of TANK1 ASODN on the tumor formation and cell apoptosis were analyzed both in SGC-7901 tumor-bearing mice and SGC-7901 cells to further elucidate the underlying mechanism. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-82/rc).

Methods

Patients and clinical specimens

This study was approved by the institutional ethics committee board of Suqian First Hospital (No. 20200093). All involved patients (n=15) were informed of the study and signed consent forms. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The anonymous medical records of the patients were reviewed, and the exclusion criteria were as follows: non-adenocarcinomas, radiotherapy or chemotherapy prior to surgery, and incomplete clinical data. Paired gastric cancer and adjacent non-tumor tissues were collected from 15 patients diagnosed with gastric adenocarcinoma at Suqian First Hospital between January 2021 and June 2021, and then preserved in liquid nitrogen.

Cell lines

We cultured SGC-7901 cells (Procell, Wuhan, China) in Roswell Park Memorial Institute (RPMI) 1640 (Procell) containing 10% fetal bovine serum (FBS; Procell) in a humidified incubator with 5% CO_2 at 37 ℃.

Tumor-bearing model

A total of 15 male BALC/C nude mice (5–6 weeks old; weighing 20±2 g, ~18–22 g) were randomly divided into 3 groups (n=5 in each group): vehicle group, TANK1 sense oligonucleotides (TANK1 SODN) group, and TANK1 ASODN group. Random numbers were generated using the standard = RAND () function in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). All animals were housed under a standardized condition with a light-dark cycle at 22±2 ℃ and 55%±5% humidity. Animal experiments were performed under a project license (No.

IACUC-20200922-01) granted by institutional ethics committee board of Zhaofenghua Biotechnology (Nanjing) Co., Ltd., in compliance with the National Institutes of Health guidelines for the care and use of animals. And the animal experiments were done in the Zhaofenghua Biotechnology (Nanjing) Co., Ltd. A protocol was prepared before the study without registration.

TANK1 SODN and TANK1 ASODN were synthesized by General Biol (General Biosystems, Anhui, China) according to the initial codon of the protein translation site of TANK1 complementary DNA (cDNA) sequence. The SGC-7901 cells in logarithmic growth phase were collected and re-suspended in normal saline. We then injected 100 μL suspension (1 million cells) subcutaneously at the right inguinal site of each nude mouse. Liposomes-encased TANK1 SODN and TANK1 ASODN were diluted with normal saline to a final concentration of 0.5 μg/μL. Each mouse was injected with 50 μL mixture for 3 times every 5 days until a tumor diameter of 0.5 cm was achieved. The vehicle group, injected with equivalent normal saline containing liposomes, was used as the negative control group. Tumor volumes were recorded every 3 days. At 24 days later, mice were sacrificed, and tumor weight was measured. Tumor volume was calculated based on the formula: tumor volume (mm^3) = (width) × $(height)^2/2$. All animals were sacrificed with an intraperitoneal injection of 200 mg/kg sodium pentobarbital (body weight). After death verification by cessation of the heartbeat, the tumor tissues were obtained for further investigation.

TUNEL staining

Formaldehyde was utilized to fix tumor tissues, and part of the tissues were embedded in paraffin. Tissue slices were prepared and stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) mixture (Roche, Shanghai, China) following deparaffinization, hydration, and permeation. Subsequently, slices were washed with phosphate-buffered saline (PBS), mixed with 3,3'-diaminobenzidine (DAB) solution, redyed with hematoxylin, and washed with flowing water. After dehydration and vitrification, the apoptotic cells were observed under microscope (Leica, Heidelberg, Germany; magnification, ×100).

Immunohistochemistry

Briefly, tissue slices were subjected to incubation with primary antibodies against Ki67 and h-TERT (Abcam, Cambridge, UK) at 4 ℃ following deparaffinization, antigen retrieval, and blockade. Then, slices were incubated with corresponding secondary antibody (Abcam). Pictures were taken under a microscope (Leica; magnification, ×100).

Real-time quantitative polymerase chain reaction

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract the total RNA, followed by reverse transcription using tRevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermantas, St. Leon-Rot, Germany). The polymerase chain reaction (PCR) was carried out using a PCR 7500 System and Power SYBR Green PCR master mix (both Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The following thermocycling conditions were used: 30 s at 95 ℃ for 1 cycle; 3 s at 95 ℃, 30 s at 60 ℃ and 30 s at 72 ℃ for 40 cycles; and 5 min at 72 ℃ for 1 cycle. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal reference gene. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Cell proliferation assay

The SGC-7901 cells in logarithmic growth phase were seeded in a 96-well culture plate. Cell proliferation ability was assessed by cell counting kit-8 (CCK-8; KeyGen, Nanjing, China) at 24, 48, and 72 h. Then, 10 μL CCK-8 solution was added to the medium and incubated for an additional 3 h. The absorbance at 450 nm was measured using a microplate reader (BioRad Laboratories, Hercules, CA, USA).

Flow cytometry analysis

Cells in each group were collected by centrifugation, washed twice with pre-cooled PBS, and then re-suspended in 500 μL buffer solution. We mixed 100 μL buffer solution with 5 μL Annexin V-APC (KeyGen), and subsequently, 5 μL propidium iodide (PI; KeyGen) was added for incubation of 15 min at room temperature (avoiding light). Next, 400 μL PBS was added to re-suspend cells and filtered with a 400-mesh sieve. Flow cytometry (Becton Dickinson and Co., Franklin Lakes, NJ, USA) was conducted to analyze apoptotic cells.

Statistical analysis

Data was represented as mean ± standard deviation and analyzed by Student's *t*-test or one-way analysis of variance

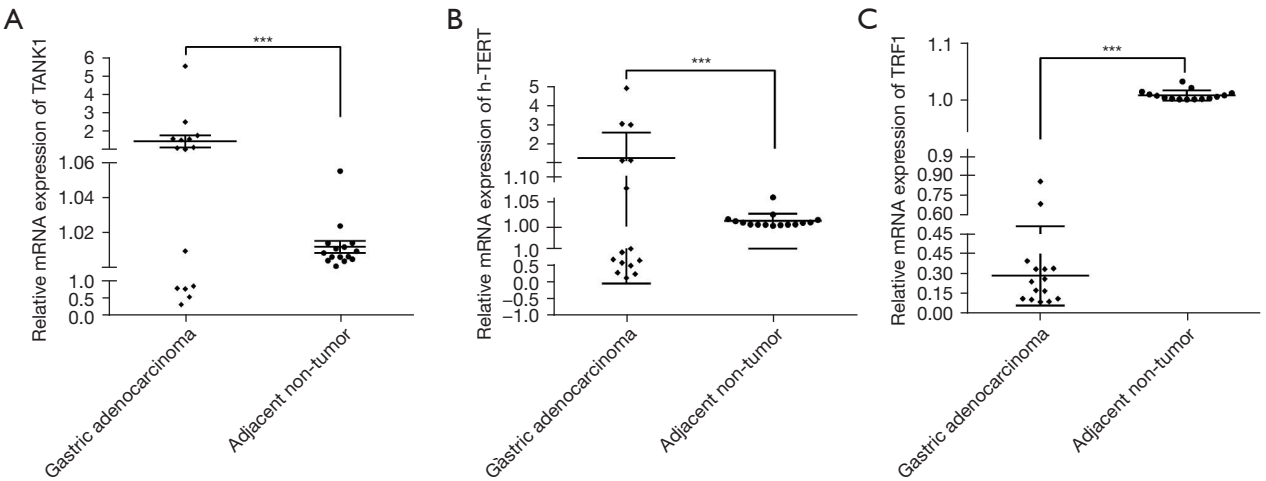

Figure 1 The expression levels of *TANK1*, *h-TERT*, and *TRF1* in gastric adenocarcinoma and adjacent non-tumor tissues. (A) The mRNA levels of *TANK1* in clinical specimens; (B) the mRNA levels of *h-TERT* in clinical specimens; (C) the mRNA levels of *TRF1* in clinical specimens. Data are represented as mean ± standard deviation, ***P<0.001. mRNA, messenger RNA.

(ANOVA) followed by Tukey's post hoc test with GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was performed at least 3 times. Statistical significance was considered when P<0.05.

Results

The expression levels of TANK1, h-TERT, and TRF1 in gastric adenocarcinoma

In order to clarify the expression levels of *TANK1*, *h-TERT*, and *TRF1*, 15 pairs of gastric adenocarcinoma and adjacent non-tumor tissues were prepared for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The results showed that the expression of *TANK1* was significantly increased in tumor tissues compared with non-tumor tissues (*Figure 1A*). Likewise, *h-TERT* was obviously elevated in tumor tissues in comparison to the adjacent non-tumor tissues (*Figure 1B*). However, it was observed that the mRNA level of *TRF1* was decreased in tumor tissues compared with non-tumor tissues (*Figure 1C*). These results indicated that the high levels *TANK1* and *h-TERT*, along with low expression of *TRF1*, were associated with the development of gastric adenocarcinoma.

TANK1 ASODN delays tumor formation and promotes cell apoptosis in SGC-7901 tumor-bearing mice

Subsequently, tumor-bearing mice were established to validate the role of *TANK1 in vivo*. We injected TANK1 SODN and TANK1 ASODN into SGC-7901 tumor-bearing mice to observe the function of *TANK1 in vivo*. Following 24 days-treatment of TANK1 SODN or TANK1 ASODN, tumor tissues from nude mice were harvested and photographed. It was obvious that TANK1 ASODN markedly lessened the tumor diameter compared to vehicle group; whereas, there were no significant distinctions between TANK1 SODN group and vehicle group (*Figure 2A*). In addition, TUNEL staining was employed to investigate the cell apoptosis in tumor tissues. The results exhibited that TANK1 ASODN prominently promoted cell apoptosis when compared with the vehicle group. Contrastingly, limited inhibition of cell apoptosis was presented in the TANK1 SODN group (*Figure 2B*). In brief, compared with TANK1 SODN, TANK1 ASODN appeared to be more effective in reducing tumor formation and enhancing cell apoptosis.

TANK1 ASODN decreases the expression levels of Ki67 and h-TERT in SGC 7901 cells tumor-bearing mice

The marker Ki67 serves as a proliferation marker, the function of which is strongly related to mitosis, and it is indispensable to tumor cell proliferation and growth (22). The expression of Ki67 is widely used as a predictive indicator for the assessment of tumor outcomes (23,24). Immunohistochemistry (IHC) assay suggested that the Ki67 positive area in the TANK1 ASODN group was dramatically reduced compared to the vehicle group and

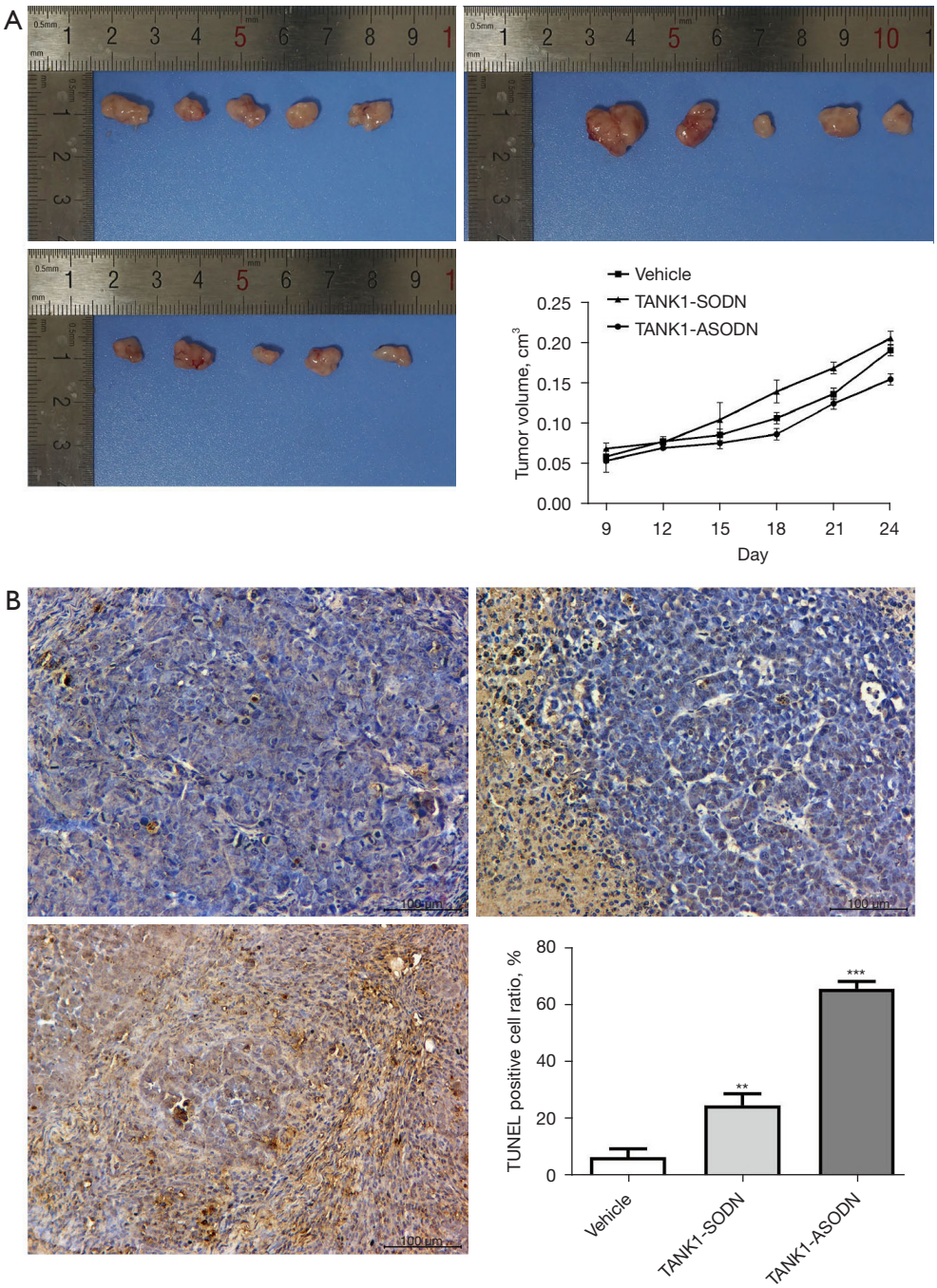

Figure 2 TANK1 ASODN delays tumor growth and promotes cell apoptosis in SGC-7901 tumor-bearing mice. (A) The photos of tumor tissues and the growth curves of SGC-7901 tumor volume growth in each group; (B) TUNEL assay was used to stain the apoptotic cells in tumor tissues. Magnification, ×100. Data are represented as mean ± standard deviation, **P<0.01, ***P<0.001 *vs.* the vehicle group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

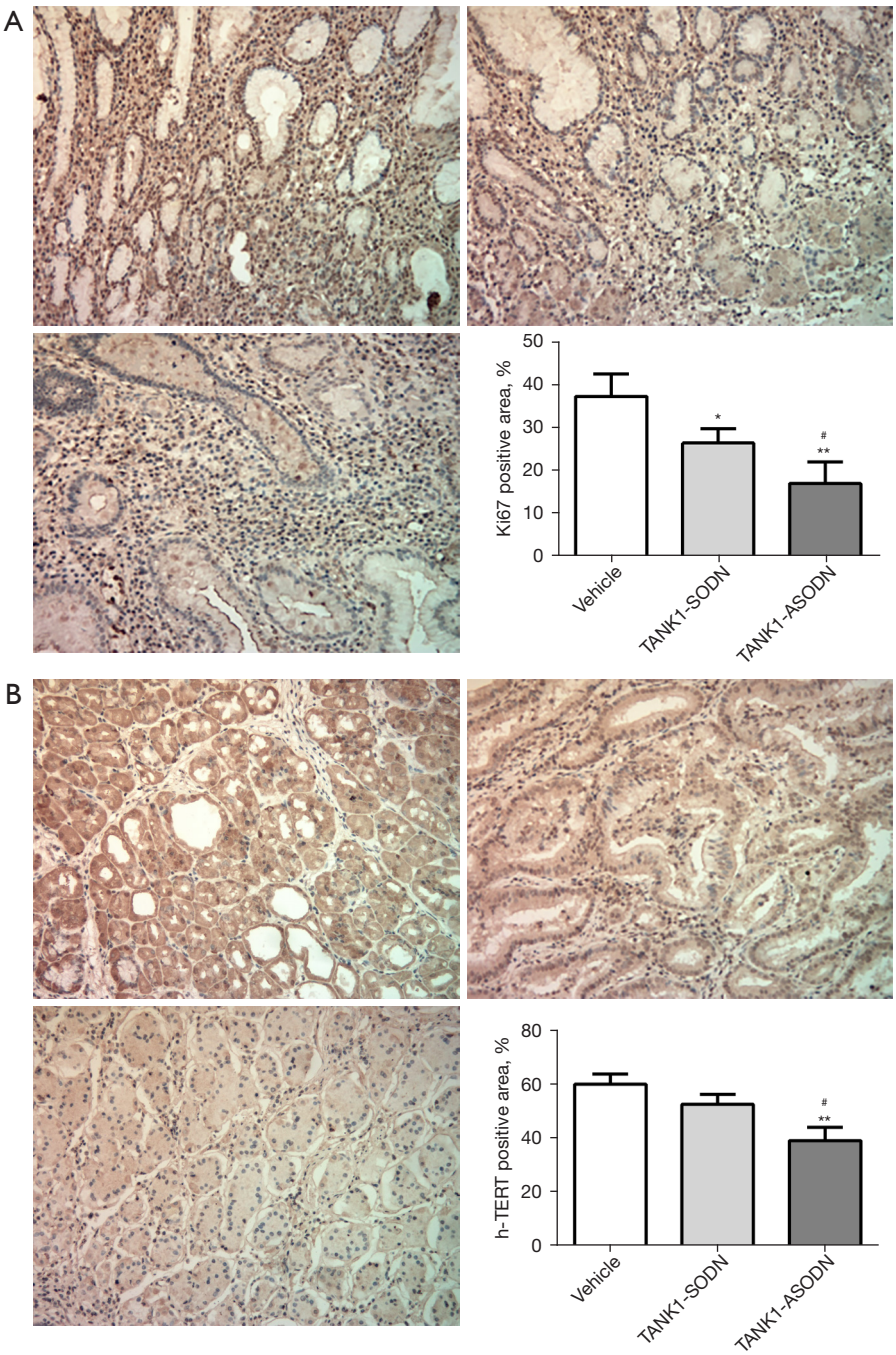

Figure 3 TANK1 ASODN delays tumor growth and increases cell apoptosis in in SGC-7901 tumor-bearing mice. (A) The distribution of Ki67 was stained by IHC assay; (B) IHC assay was used for measurement of *h-TERT* expression. Magnification, ×100. Data is represented as mean ± standard deviation, *P<0.05, **P<0.01 *vs.* the vehicle group; #P<0.05 *vs.* the TANK1 SODN group. IHC, immunohistochemistry.

TANK1 SODN groups (*Figure 3A*). Of note, TANK1 SODN also decreased the expression of Ki67 (*Figure 3A*). Moreover, the level of *h-TERT* was estimated, which indicated that TANK1 ASODN remarkably restrained the expression of *h-TERT* relative to the vehicle group and TANK1 SODN group (*Figure 3B*). These results illustrated that TANK1 ASODN could limit tumor growth and decrease the expression of *h-TERT*.

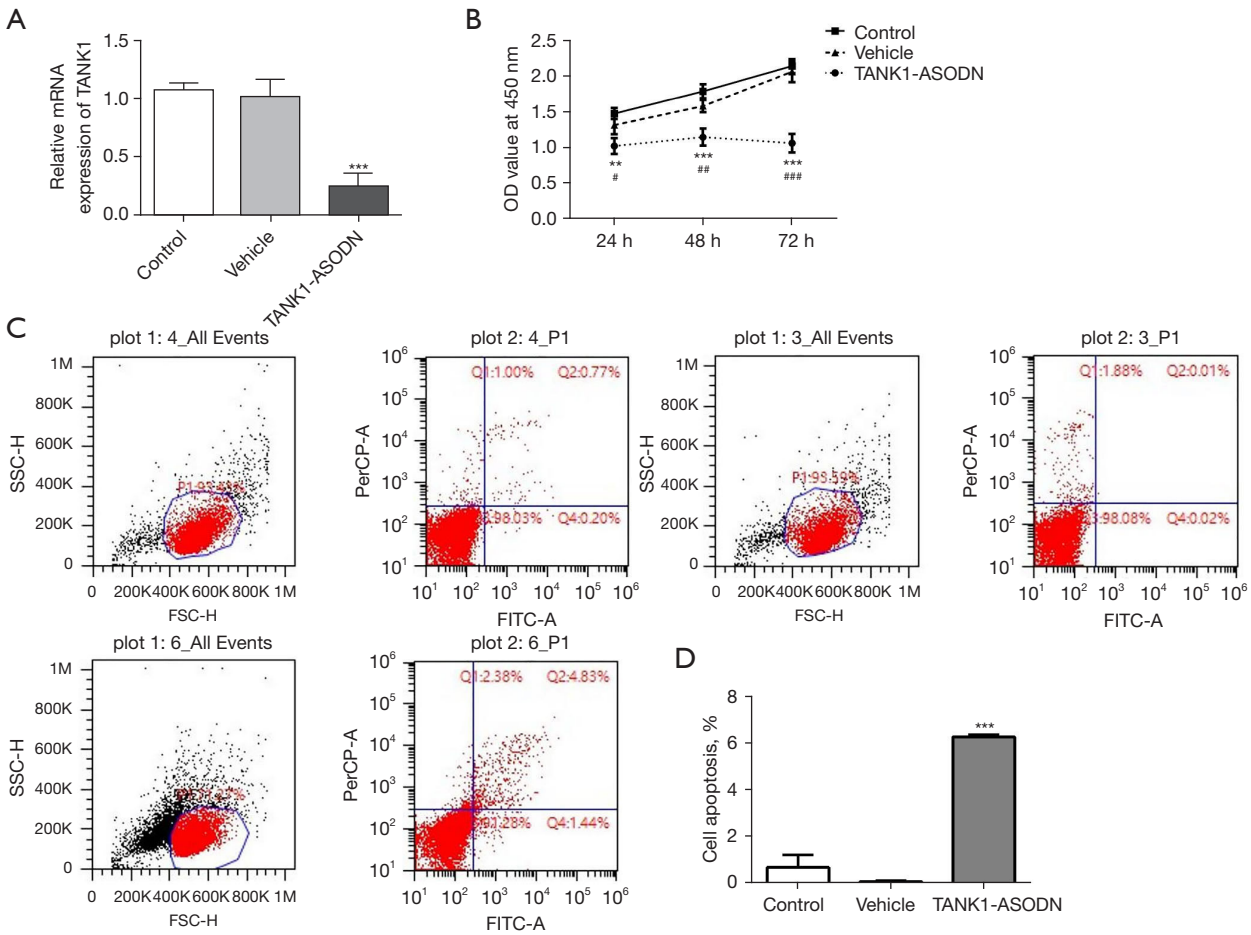

Figure 4 Silencing *TANK1* inhibits the proliferation and promotes the apoptosis of gastric adenocarcinoma cells. (A) RT-qPCR was conducted to investigate the mRNA levels of TANK1; (B) cell viability was measured by CCK-8 assay; (C,D) flow cytometry analysis was utilized to examine apoptotic cells. Data are represented as mean ± standard deviation, **P<0.01, ***P<0.001 *vs.* the vehicle group; #P<0.05, ##P<0.01, ###P<0.001 *vs.* the Control group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; mRNA, messenger RNA; CCK-8, cell counting Kit-8.

Silencing TANK1 inhibits the proliferation and promotes the apoptosis of SGC-7901 cells

To investigate the function of *TANK1* in gastric adenocarcinoma *in vitro*, TANK1 ASODN was transfected into SGC-7901 cells. As shown in *Figure 4A*, TANK1 ASODN inhibited the expression of *TANK1* as compared to the vehicle group. The CCK-8 assay suggested that TANK1 ASODN remarkably inhibited proliferation of SGC-7901 cells compared with the vehicle group (*Figure 4B*). To further investigate the effect of TANK1 ASODN on the apoptosis of SGC-7901 cells, flow cytometry analysis was conducted. The results revealed that the apoptotic rate of cells transfected with TANK1 ASODN was apparently higher than control group or vehicle group (*Figure 4C,4D*). Collectively, TANK1 ASODN exhibited a powerfully suppressive effect on cell proliferation and a significantly promotive effect on cell apoptosis.

Knockdown of TANK1 affects the expression levels of h-TERT and TRF1 in SGC-7901 cells

After the above exploration, the possible mechanism was further probed. The *TANK1* gene could weaken the binding of *TRF1* to telomeres, which provides a premise for the activation of telomerase and its binding with telomere (9). The *TRF1* gene is an inhibitor of telomere elongation and related to the negative feedback mechanism of stabilizing

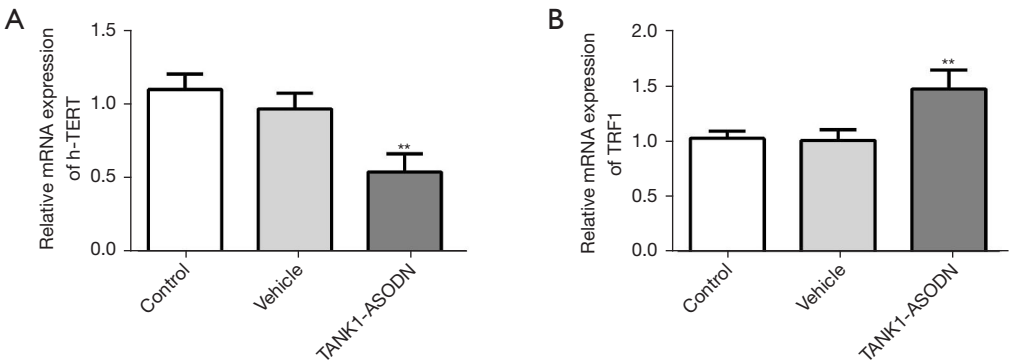

Figure 5 Knockdown of *TANK1* affects the expression levels of *h-TERT* and *TRF1*. (A) The relative expression of *h-TERT* was evaluated by RT-qPCR; (B) RT-qPCR was performed to estimate the mRNA levels of *TRF1*. Data was represented as mean ± standard deviation, **P<0.01 *vs.* the vehicle group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; mRNA, messenger RNA.

telomere length. When compared to the vehicle group, the expression of *h-TERT* was decreased in the TANK1 ASODN group (*Figure 5A*). Moreover, it was noted that TANK1 ASODN simultaneously elevated the mRNA level of *TRF1* compared with the vehicle group (*Figure 5B*). Taken together, these results indicated that TANK1 ASODN could downregulate but upregulate the mRNA levels of *h-TERT* and *TRF1*, respectively.

Discussion

Telomere, telomerase, and telomere binding proteins play pivotal roles in cellular immortality and tumorigenesis. The gene *TANK1* is a regulator of telomerase activation and telomere prolongation (25). The level of TANK negatively correlates with poor survival of lung cancer patients (26). Recent studies have demonstrated that inhibition of TANK significantly reduces prostate cancer cell proliferation and inhibits the growth of human osteosarcoma xenograft (27,28). Besides, TANK promotes proliferation of ovarian cancer–through activation of Wnt/β-catenin signaling (29). However, the expression of *TANK1* in gastric cancer is still controversial. A previous study suggested that *TANK1* is significantly upregulated in gastric cancer tissues (20), while contrary findings were revealed by Yamada *et al.* (13). The most common type of gastric cancer is adenocarcinoma. In the present study, 15 matched gastric adenocarcinoma and adjacent non-tumor tissues were collected according to the exclusion criteria. After detection, we disclosed that the mRNA levels of *TANK1* and *h-TERT* were elevated while *TRF1* was downregulated in gastric adenocarcinoma tissues, together indicating that the overexpression of *TANK1* may be strongly correlated with gastric carcinogenesis.

To further evaluate the specific functions of *TANK1* on gastric adenocarcinoma, a tumor-bearing model was established by injection with TANK1 ASODN. It was observed that TANK1 ASODN inhibited the progression of gastric adenocarcinoma and affected the expression of *h-TERT*. Furthermore, SGC-7901 cells were cultured *in vivo* and transfected with TANK1 ASODN. The cell proliferation was markedly inhibited, but the cell apoptosis was significantly increased by TANK1 ASODN. It has been reported that TANK knockdown using small interfering RNA (siRNA) could suppress the proliferation of the hepatocellular cancer (HCC) cell lines (30). The proliferation of human tumor cells is antagonized by TANK inhibitors via stabilization of angiomotin (31). In addition, TANK inhibition impairs directional migration and invasion of lung cancer cells (32). These findings highlight an anti-tumor effect of TANK inhibitors, consistent with the results presented in the current study.

In conclusion, the present study illustrated that *TANK1* was increased in gastric adenocarcinoma, and TANK1 ASODN developed an anti-tumor role possibly through manipulating the expressions of *h-TERT* and *TRF1*. However, telomerase activity and telomere length were not included in this study, and to improve the research, further investigation is indispensable.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-82/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-82/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-82/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the institutional ethics committee board of Suqian First Hospital (No. 20200093). All involved patients (n=15) were informed of the study and signed consent forms. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Animal experiments were performed under a project license (No. IACUC-20200922-01) granted by institutional ethics committee board of Zhaofenghua Biotechnology (Nanjing) Co., Ltd., in compliance with the National Institutes of Health guidelines for the care and use of animals.

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