JBUON 2021; 26(5): 1842-1849
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Trefoil factor 3 silencing can inhibit the proliferation and apoptosis of lung cancer cells

Moran Liu[1], Yin Xiao[2]

[1]Laboratory Medicine, The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, China. [2]Department of Medical Oncology, The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, China.

Summary

Purpose: *At present, the global incidence of lung cancer is still high. Exploring its effective treatment is still a crucial research direction. Trefoil factor 3 (TFF3) was found to be related to the proliferation and apoptosis of many tumor cells. Therefore, this article focuses on the effect of TFF3 on SPC-A1 lung cancer cells.*

Methods: *The tissue samples of lung cancer patients were collected, and the expression level of TFF3 was detected by Western blot (WB) and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) techniques. After transfection technique was used to silence the expression of TFF3 in SPC-A1 cells, the proliferation activity of SPC-A1 cells was detected by CCK-8 assay and EdU staining, and the cell cycle and related factor expression levels were also detected. The apoptosis rate of SPC-A1 cells was detected by Tunel staining and flow cytometry, and the expression levels of apoptosis-related factors were also detected.*

Results: *TFF3 in lung cancer tissues was obviously higher than that in para-carcinoma tissue. At the same time, similar results were found in SPC-A1 lung cancer cells. CCK-8 assay and EdU staining found that silencing TFF3 gene expression can effectively inhibit the proliferation of SPC-A1 cells. Flow cytometry detection of SPC-A1 cell cycle showed that cells were blocked in G0/G1 phase, and the number of cells in S+G2/M phase was obviously reduced. Cyclin D1 expression was also obviously reduced. At the same time, silencing TFF3 gene expression can promote the increase of Bax expression and inhibit the expression of Bcl-2, thereby increasing the apoptosis rate of SPC-A1 cells. Furthermore, silencing the TFF3 gene can effectively inhibit the excessive activation of the Wnt/β-catenin pathway in SPC-A1 cells.*

Conclusions: *Our results show that the expression of TFF3 in lung cancer was obviously increased. Silencing TFF3 in SPC-A1 cells can inhibit the cell proliferation and promote cell apoptosis. At the same time, we confirmed that silencing TFF3 gene can inhibit the abnormal activation of Wnt/β-catenin signaling pathway in SPC-A1 cells.*

Key words: *lung cancer, adenocarcinoma, Trefoil factor 3, Wnt/β-catenin signaling pathway, proliferation, apoptosis*

Introduction

Lung cancer is not only the most common cancer in China, but also the most common cancer in the world. In China, with the acceleration of industrialization, increased environmental pollution, and a widespread smoking population, the cancer burden of lung cancer is increasing. Although the 5-year survival rate of lung cancer has increased, it is still less than 20% [1,2]. Among the pathological types of lung cancer, non-small cell lung cancer (NSCLC) accounts for about 8.5%, and most patients with NSCLC have already found local lymph node and vascular invasion and distant metastasis of lymph nodes and other organs at the first visit. These patients are in the advanced stages of lung cancer, thus losing the opportunity for surgery [3]. Although the rate of early diagnosis of lung cancer has

Corresponding author: Yin Xiao, MM. Department of Medical Oncology, The Third Affiliated Hospital of Qiqihar Medical University, 27 Taishun Street, Tiefeng District, Qiqihar, Heilongjiang 161000, China.
Tel: +860452-2697459; Email: 47638423@qq.com
Received: 15/07/2021; Accepted: 11/08/2021

increased in recent years, a considerable portion of lung cancer patients are already found in advanced stages, so new treatment options must be explored.

Trefoil factor 3 (TFF3) is the latest small-molecule secreted polypeptide found in the TFF family. It is a clover domain composed of 42 amino acid residues folded by disulfide bonds [4]. There is increasing evidence that TFF3 plays a crucial role in the occurrence and progression of cancer. It has been found that TFF3 mRNA and protein are overexpressed in different solid tumors, and promote cancer cell migration, invasion, proliferation, survival and angiogenesis [5-8]. Studies have confirmed that TFF3 can promote cell dispersion by reducing cell-cell or cell-matrix interactions, further improving cell migration. However, the specific mechanism by which TFF3 promotes lung cancer proliferation and apoptosis is not clear.

The Wnt/β-catenin signaling pathway plays an important role in the regulation of various biological processes, including cell proliferation, embryonic growth, development, and maintenance of tissue homeostasis [9,10]. When the Wnt/β-catenin signaling pathway is activated by exogenous signals, the phosphorylation level of downstream core effector protein β-catenin is reduced, thereby preventing its recognition by β-TROP and degradation by the proteasome. When β-catenin accumulates in the cytoplasm to a certain concentration, it migrates into the nucleus and binds to the TCF/LEFs family of transcription factors in the nucleus, causing the activation of downstream genes and causing abnormal cell proliferation [11]. The Wnt/β-catenin signaling pathway is off and maintains a low level of intracellular β-catenin in normal somatic cells. Researches have shown that the excessive activation of the Wnt/β-catenin signaling pathway is associated with the occurrence of various tumors including nodular lung cancer, gastric cancer, breast cancer, etc. [12,13]. At the same time, previous research found that the positive expression rate of β-catenin in lung cancer tissue was higher than that in normal lung tissue, indicating that abnormal expression of β-catenin is associated with the progression of lung cancer.

To this end, we selected SPC-A1 lung cancer cells as a research object to explore the effect of TFF3 on SPC-A1 cell proliferation and apoptosis.

Methods

Surgical specimen collection

According to WHO histopathological classification criteria, 20 cases of adenocarcinoma (12 male and 8 female), aged 48-76 years, were selected after patients' informed consent. In each case, 100 mg of typical cancer tissue and tissue 1 cm away from the cancer were frozen in liquid nitrogen. The above cases were confirmed by two pathologists.

Cell culture

The lung cancer cells (American type culture collection, Manassas, VA, USA) growth status was observed under an inverted microscope, and the cells were passed when they reached 80% of the bottom of the dish. The culture medium in the Petri dish was discarded, phosphate-buffered saline (PBS) was used to wash for 3 times, 1 mL of 0.25% pancreatin (Life Technology, Wuhan, China) was added to make it evenly cover the surface of the growing cells for digestion. Then, 2 mL of complete medium (Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) containing 10% fetal bovine serum (FBS; Life Technology, Wuhan, China)) was added to stop the digestion, and removed the cells to 1:3 ratio inoculated in two other cell culture dishes.

Plasmid transfection

The day before transfection, about 5×10^5 of SPC-A1 cells were planted in six-well plates. When the cell fusion rate reached 70% on the second day, it was ready for transfection. First, shRNA was added to 100 μL Opti-MEM, mixed gently and left at room temperature for 5 min. A certain proportion of Lipofectamine^{2000} (Kai Ji, Nanjing, China) was added to 100 μL Opti-MEM, mixed gently and left at room temperature for 5 min. The two were mixed at room temperature for 20 min to form a shRNA/Lipofectamine^{2000} mixture. After aspirating the culture medium from the culture dish, 2 mL Opti-MEM was added to each well, and the transfection mixture was added to the culture wells containing the cells and culture medium. The culture dish was placed in a 37°C, 5% CO_2 incubator for about 6 h and then changed the medium.

CCK-8 assay

The lung cancer cells with good growth conditions were inoculated in 96-well plates with 5000/ well. When the cell fusion reached 70-80%, they were transfected by the above method. Then, the culture plate was placed into the incubator to continue culturing for 24, 48 and 72 h. After reaching the incubation time, 10 μL of CCK-8 reagent (Jian Cheng, Nanjing, China) was added to each well, and the plates were placed in the incubator to continue incubation for another 2 h. The absorbance of each well was measured with a microplate reader (Elabscience, Wuhan, China) at 450nm. Cell proliferation OD value of each group = OD value of experimental well/ OD value of blank control well.

Western blotting

The total protein of tissues and cells was extracted with protein lysate (Camilo Biological, Nanjing, China), the protein lysate was collected, and the supernatant was collected after centrifugation at 13000 r/min for 5 min at 4°C. The bicinchoninic acid (BCA, Camilo Biological, Nanjing, China) kit quantified the protein and adjusted the protein concentration to keep it consistent. After boiling, the protein was stored at -20°C. We used 10% sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis (SDS-PAGE), and the protein was transferred to a polyvinylidene difluoride (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) membrane. The membrane was blocked with 5% skimmed milk powder for 2 h, diluted the primary antibody (TFF3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Cyclin D1, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; Bcl-2, Abcam, Cambridge, MA, USA, Mouse, 1:2000; Bax, Abcam, Cambridge, MA, USA, Mouse, 1:2000; β-catenin, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; p-β-catenin, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; GAPDH, Proteintech, Rosemont, IL, USA, 1:5000) in the corresponding proportion, incubated overnight at 4°C, TBST buffer was used to wash the membrane, and the membrane was incubated with secondary antibody (goat anti-rabbit IgG antibody, Yifei Xue Biotechnology, Nanjing, China, 1:2000) at room temperature for 1 h. After washing the membrane with TBST buffer, the developer was used for development.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using total RNA extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcription was performed using PrimeScript^{RT} reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) to prepare cDNA, and each cDNA sample was subjected to qRT-PCR reaction with the following primers. The reaction conditions were: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 10 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec, and 40 cycles. The primer sequences are shown in Table 1 below. The $2^{-\Delta\Delta Ct}$ method was used for statistical analysis. Primers used are shown in Table 1.

Flow cytometry detection of cell cycle

The SPC-A1 cells after transfection were taken, digested with trypsin, and then the cells were collected. The cells were stored in 75% ethanol solution at -20°C overnight. The cells were then sent to the flow center for detection by the person in charge of the center.

Flow cytometry detection of cell apoptosis

After transfection the SPC-A1 cells were taken, digested with trypsin without EDTA, collected and adjusted the final cell concentration to 1×10^6 cells/mL. Then, were stained according to the Annexin V-FITC/PI kit (Kai Ji, Nanjing, China) instructions. After incubation for 10 min in the dark apoptosis was detected by flow cytometry (Becton Dickinson, Heidelberg, Germany). Annexin V-FITC positive cells were judged as apoptotic cells.

EdU staining

After transfection the SPC-A1 cells were taken and diluted in the EdU solution according to the instructions of the EdU staining kit (BD Pharmingen, Shanghai, China). The diluted EdU solution was added to each well and

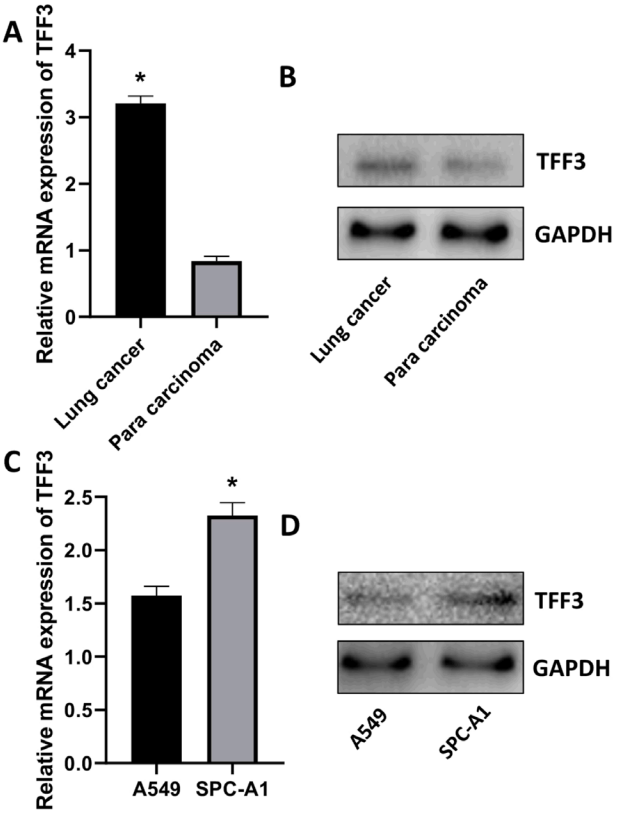

Figure 1. TFF3 expression was up-regulated in lung cancer tissues and cells. **A:** Western blot was used to detected the protein expression of TFF3 in lung cancer tissues and para-carcinoma tissue. **B:** qRT-PCR was used to detected the mRNA expression of TFF3 in lung cancer tissues and para-carcinoma tissue (*indicates statistical difference from the para-carcinoma tissue $p<0.05$). **C:** Western blot was used to detected the protein expression of TFF3 in A549 and SPC-A1 lung cancer cell. **D:** qRT-PCR was used to detected the mRNA expression of TFF3 in A549 and SPC-A1 lung cancer cell. (*indicates statistical difference from the A549 group $p<0.05$).

Table 1. Real time PCR primers

Gene name	*Forward (5'>3')*	*Reverse (5'>3')*
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
TFF3	CTCCTCTTCGCACTTCTGCTC	TACACTGCTCCGATGTGACAG
Cyclin D1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR: quantitative reverse-transcription polymerase chain reaction

incubated overnight. After the incubation, the cells were washed twice with PBS. 4% paraformaldehyde was used to fix the cells, TrixonX-100 (Yifei Xue Biotechnology, Nanjing, China) was used to break the cell membrane, and then incubated with EdU reagent for 30 minutes at room temperature. After staining the nucleus with DAPI reagent, the 6-well plates were placed under a fluorescent inverted microscope to observe and take pictures.

Tunel staining

After taking transfected SPC-A1 cells, 4% paraformaldehyde was used to fix the cells. TrixonX-100 was used for cell membrane rupture. Then, the Tunel kit (BD Pharmingen, Shanghai, China) instructions were used to carry out the labeling reaction. After the reaction, PBS was used to wash the cells, and the 6-well plates were placed under a fluorescent inverted microscope to observe and take pictures.

Statistics

SPSS21.0 software was used for statistical analyses. The experimental data was expressed as mean ± standard deviation. The measurement data between groups were analyzed by ANOVA and q test. P<0.05 was considered statistically significant.

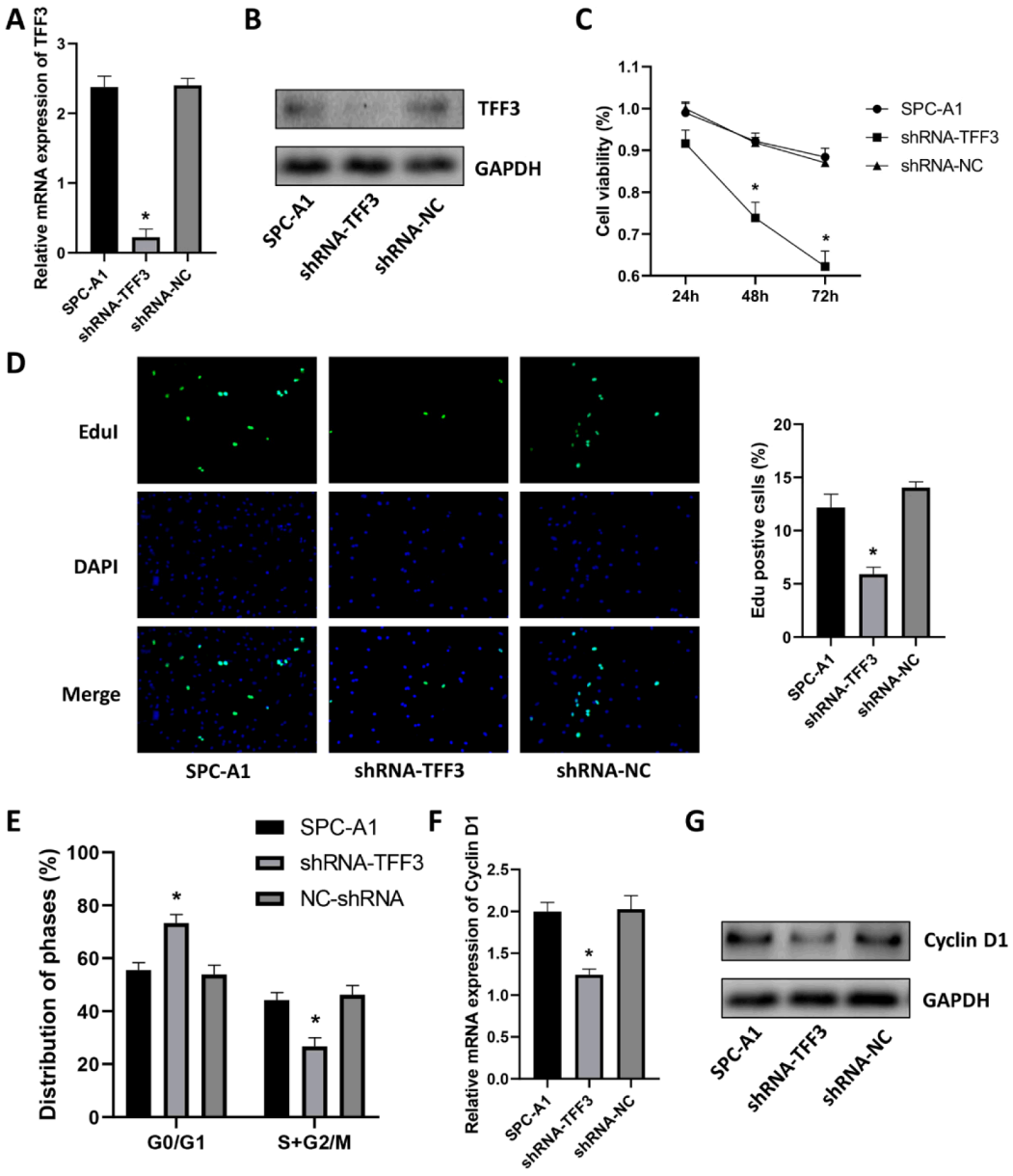

Figure 2. Silencing TFF3 expression can inhibit the proliferation of SPC-A1 lung cancer cells. **A:** qRT-PCR was used to detected the mRNA expression of TFF3 in SPC-A1 cell. **B:** Western blot was used to detected the protein expression of TFF3 in SPC-A1 cell. **C:** CCK-8 assay was used to detected the activity of SPC-A1 cell. **D:** EdU staining was used to detected the proliferation of SPC-A1. **E:** Flow cytometry was used to detect the SPC-A1 cycle. **F:** qRT-PCR was used to detected the mRNA expression of Cyclin D1 in SPC-A1 cell. **G:** Western blot was used to detected the protein expression of Cyclin D1 in SPC-A1 cell. (*indicates statistical difference from the SPC-A1 and NC-shRNA group (p<0.05).

Results

TFF3 expression was up-regulated in lung cancer tissues and cells

We collected 20 cases of lung cancer tumor tissues and their corresponding para-carcinoma tissue from our hospital. We applied Western blot (WB) and qRT-PCR methods to detect TFF3 expression in these tissues. Both results confirmed that TFF3 expression was up-regulated in lung cancer tissues (Figure 1A and 1B). In order to further explore the biological function of TFF3 on lung cancer cell we used WB and qRT-PCR methods to detect the expression of TFF3 in A549 and SPC-A1 lung cancer cell lines (Figure 1C and 1D). We found that TFF3 was highly expressed in SPC-A1 cells, while TFF3

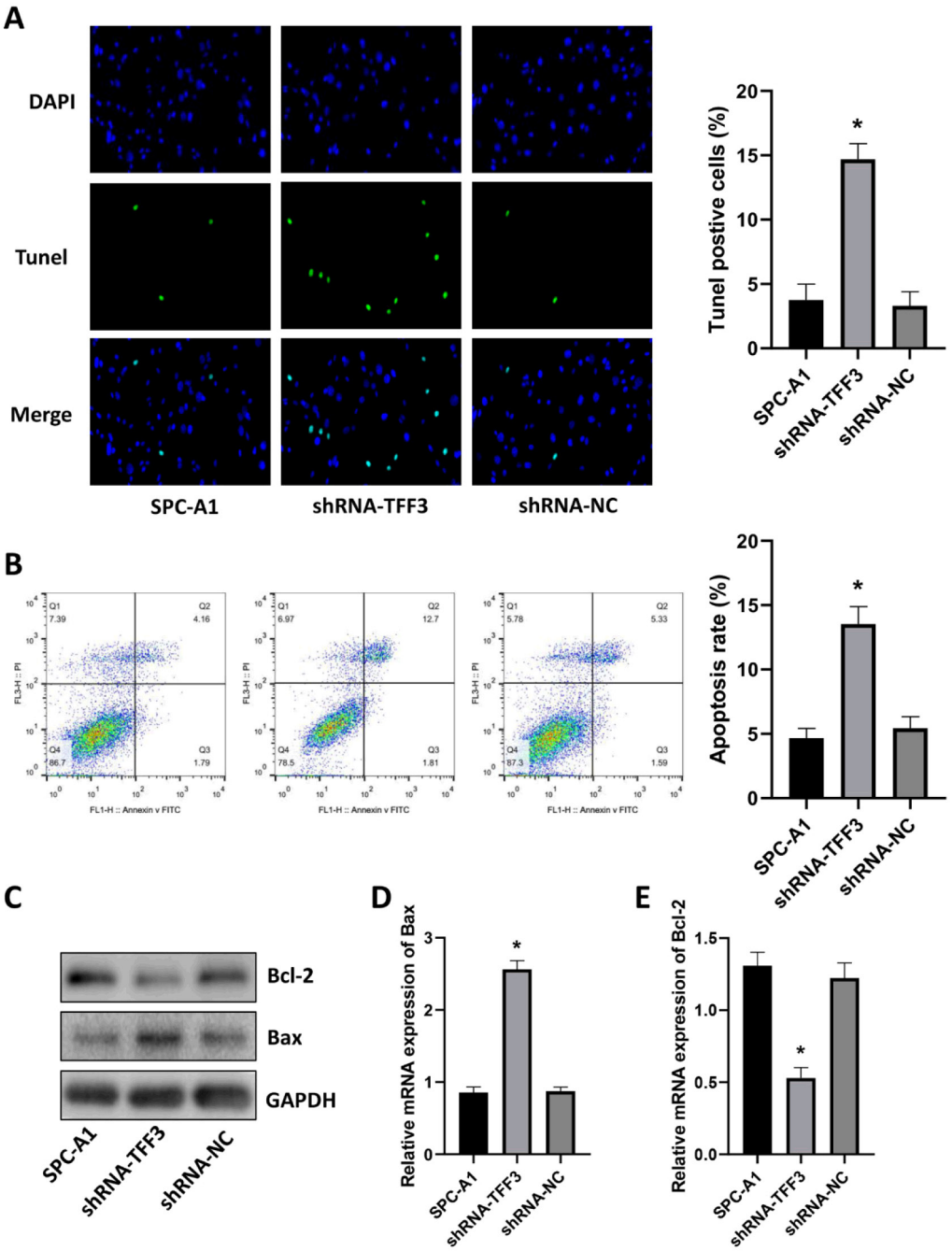

Figure 3. Silencing TFF3 expression can promote apoptosis of SPC-A1 lung cancer cells. **A:** Tunel staining was used to detected the apoptosis level of SPC-A1. **B:** Flow cytometry was used to detect the SPC-A1 apoptosis rate. **C:** Western blot was used to detected the protein expression of Bax and Bcl-2 in SPC-A1 cell. **D** and **E:** qRT-PCR was used to detect the mRNA expression of Bax and Bcl-2 in SPC-A1 cell. (*indicates statistical difference from the SPC-A1 and NC-shRNA group ($p<0.05$).

was low in A549 cells. According to the expression of TFF3 on lung cancer cell lines, SPC-A1 cell line was our main research object.

Silencing TFF3 expression can inhibit the proliferation of SPC-A1 lung cancer cells

First, we transfected synthetic TFF3 shRNA into SPC-A1 cells, and qRT-PCR results showed that TFF3 mRNA expression in SPC-A1 cells of TFF3 shRNA group decreased significantly (Figure 2A). WB results also confirmed that TFF3 shRNA can obviously inhibit TFF3 protein expression (Figure 2B). Next, the CCK-8 assay was used to detect the cell proliferation of 3 groups (Figure 2C). We found that the cell activity of the SPC-A1 and NC-shRNA groups was obviously higher than that of the TFF3 shRNA group. Then, EdU staining detected the proliferation of SPC-A1 cells and found that the proliferation rate of TFF3 shRNA group decreased obviously (Figure 2D). In addition, flow cytometry detection of the cell cycle found that SPC-A1 cells in the TFF3 shRNA group were blocked in the G0/G1 phase, and the number of cells in the S+G2/M phase was obviously reduced (Figure 2E). At the same time, qRT-PCR detection of Cyclin D1 mRNA in 3 groups of cells found that the expression of Cyclin D1 mRNA in the TFF3 shRNA group was decreased (Figure 2F) and the WB result was similar to the qRT-PCR result (Figure 2G). The above results indicated that silencing TFF3 can effectively inhibit the proliferation of SPC-A1 lung cancer cells.

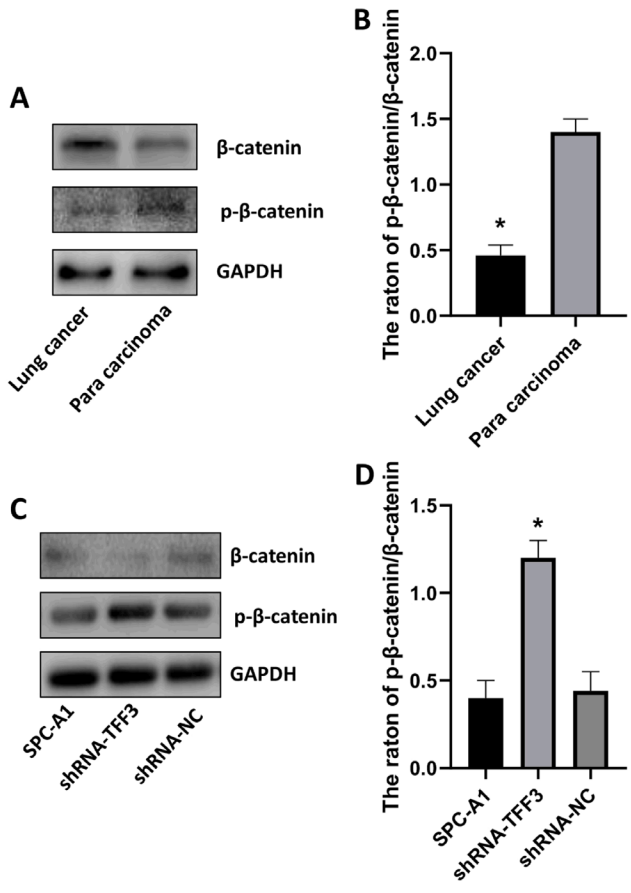

Figure 4. Silent TFF3 expression can inhibit excessive activation of Wnt/β-catenin signaling pathway. **A:** Western blot was used to detected the protein expression of β-catenin and p-β-catenin in lung cancer tissues and para-carcinoma tissue. **B:** The ratio of protein gray value analysis. (*indicates statistical difference from the para-carcinoma tissue $p<0.05$). **C:** Western blot was used to detected the protein expression of β-catenin and p-β-catenin in SPC-A1 cell. **D:** The ratio of protein gray value analysis. (*indicates statistical difference from the SPC-A1 and NC-shRNA group ($p<0.05$).

Silencing TFF3 expression can promote apoptosis of SPC-A1 lung cancer cells

In order to further explore the role of TFF3 in regulating apoptosis of SPC-A1 cells we first used Tunel staining to detect the level of apoptosis in each group (Figure 3A). The results showed that silencing TFF3 expression can obviously promote apoptosis of SPC-A1 cells. At the same time, the detection of apoptosis rate by flow cytometry also confirmed the former results (Figure 3B). In addition, WB detected the expression of Bax and Bcl-2. The results showed that when the TFF3 gene of SPC-A1 cells was silenced, the expression of Bax increased obviously, and the expression of Bcl-2 decreased (Figure 3C). Moreover, qRT-PCR also obtained similar results as WB (Figure 3D and 3E).

Silent TFF3 expression can inhibit excessive activation of Wnt/β-catenin signaling pathway

First, we detected the expression of β-catenin and the phosphorylation level in lung cancer and para-carcinoma tissues by WB (Figure 4A and 4B). The results showed that the expression of β-catenin in lung cancer tissues was obviously up-regulated, while the expression of p-β-catenin was obviously reduced. In *in vitro* studies the results showed that after silencing TFF3 expression, the expression of β-catenin in SP-A1 cells was obviously inhibited, while p-β-catenin expression increased obviously (Figure 4C and 4D). From this, we speculated that silencing TFF3 expression can inhibit excessive activation of Wnt/β-catenin signaling pathway in SPC-A1 cells.

Discussion

Lung adenocarcinoma is a very common pathological type in lung cancer. In early reports, adenocarcinoma accounted for 40% of all lung cancer types, but the incidence of adenocarcinoma has increased in recent years. In Asian populations, the incidence of lung adenocarcinoma has

shown an upward trend compared with other types. And most of the patients with lung cancer who visited the hospital were already in advanced stage at the first visit, and lost the best treatment opportunity.

The emergence of molecular targeted drugs has brought hope to patients with advanced lung cancer, while the current main molecular targeted drugs are concentrated in lung adenocarcinoma. However, due to the resistance of tumor cells to drugs, the anti-tumor effects of many chemotherapeutics have been suppressed [14]. Therefore, we hope to find new targets to fight lung cancer cells. After that, we collected 20 cases of lung cancer tumor tissues and their para-carcinoma tissue, and after testing it was confirmed that TFF3 expression in tumor tissues increased obviously. At the same time, as confirmed by previous studies, TFF3 can promote cancer cell migration, invasion, proliferation, survival and angiogenesis. From this we speculated that TFF3 has a key role in the generation and treatment of lung cancer.

At the same time, we also detected TFF3 expression levels in A549 and SPC-A1 cell lines, and the results indicated that TFF3 expression was higher in SPC-A1 cells. Therefore, we selected SPC-A1 cells for subsequent experimental verification. In this experiment, we successfully constructed a SPC-A1 cell line that stably knocked down TFF3 expression by plasmid transfection technology. qRT-PCR and WB verified the silencing efficiency of stable transfection. The proliferation of the 3 groups of cells was compared by CCK-8 assay and EdU proliferation staining. It was found that the proliferation rate of SPC-A1 cells in the untransfected group or NC group was high. After TFF3 gene was silenced, the proliferation ability of SPC-A1 cells was significantly reduced. At the same time, flow cytometry also found that when the TFF3 gene of SPC-A1 cells was silenced, the number of cells in S+G2/M phase was obviously reduced, and the cells were blocked in G0/G1 phase. Cyclin D1 is a key protein that regulates G1 phase. In G1 phase, it can be combined with CDK4/6 to activate the kinase activity of CDK4/6, driving cells into S phase to promote cell proliferation [15,16]. As a proto-oncogene, the abnormality of Cyclin D1 is closely related to the uncontrolled cell proliferation caused by the cell cycle disorder during tumor development. It has also been confirmed in gliomas that by silencing or inhibiting Gli 1 expression, Cyclin D1 level can be down-regulated and tumor cell proliferation can be inhibited [17]. At the same time, we also found that Cyclin D1 was down-regulated in SPC-A1 cells after silencing TFF3 gene expression. The above results suggested that after TFF3 gene silencing, the proliferation ability of SPC-A1 cells was obviously reduced, which indirectly reflects the role of TFF3 as a pro-tumor factor in SPC-A1 cells.

Apoptosis is a normal process of body cells, and it is the active programmed death of cells under the control of genes. Among them, Bcl-2 family and Caspase family play a very key role in the signal transduction of apoptosis [18,19]. Once the expression of the above genes or proteins is abnormal, the normal apoptosis of the cells will be inhibited, thereby promoting the occurrence and development of malignant tumors. As one of the most important oncogenes in apoptosis, Bcl-2 is also a key driver gene for the development and progression of various malignant tumors. It regulates apoptosis mainly by acting on mitochondria and endoplasmic reticulum, and inhibits tumor cell apoptosis caused by chemotherapy drugs. Bax is the most important pro-apoptotic protein in the Bcl-2 family and can bind to Bcl-2 to form a heterodimer. However, if Bax is in a high expression state, Bax itself can form a Bax/Bax homodimer structure, thereby promoting apoptosis. Our results found that in SPC-A1 cells, Bax expression was obviously down-regulated, while Bcl-2 expression was obviously up-regulated, thereby inhibiting SPC-A1 cell apoptosis. After silencing TFF3 gene expression, Bax expression was obviously up-regulated, and Bcl-2 expression was also suppressed, thereby promoting apoptosis of SPC-A1 cells. The above results indirectly reflect that TFF3 plays an anti-apoptotic role in SPC-A1 cells.

The Wnt/β-catenin signaling pathway is also a very conservative pathway in biological evolution. The abnormal activation of Wnt signal transduction pathway is related to the occurrence and invasion of cell carcinogenesis. Among them, β-catenin is the core effector molecule of Wnt/β-catenin signaling pathway, and its translocation from cytoplasm to nucleus is considered to be a sign of its biological function after the signaling pathway is activated [20]. Our research found that the expression of β-catenin in lung cancer tissues was obviously up-regulated, while the expression of p-β-catenin was obviously down-regulated. At the same time, *in vitro* experiments also confirmed that the expression of β-catenin and phosphorylation level had similar effects in SPC-A1 cells, and showed the opposite trend after silencing TFF3 gene. From this, we speculated that the interaction between TFF3 and Wnt/β-catenin signaling pathway in lung cancer cells allowed to better understand the mechanism by which tumor cells rely on this signaling pathway to benefit their own survival and proliferation.

Conclusions

This study mainly explored the biological role and regulatory mechanism of TFF3 in lung cancer. Our research showed that the expression of TFF3 in lung cancer was significantly increased. At the molecular level, silencing the expression of TFF3 in SPC-A1 cells could inhibit the cell proliferation and promote cell apoptosis. At the same time, we confirmed that silencing TFF3 gene can inhibit the Wnt/β-catenin signaling pathway abnormal activation. Therefore, TFF3 plays a crucial role in the occurrence and development of lung cancer, and may be a potential target for the treatment of this disease.

Funding acknowledgements

Social Development Project of Qiqihar Science and Technology Bureau (SFZD-2015029).

Conflict of interests

The authors declare no conflict of interests.

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