

# Toosendanin inhibits growth and induces apoptosis in colorectal cancer cells through suppression of AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway

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**Abstract.** AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling pathway plays an important role in the progression of colorectal cancer (CRC). Toosendanin (TSN) is a triterpenoid extracted from the bark or fruits of *Melia toosendan* Sieb et Zucc and possesses anti-tumour effects on various human cancer cells. However, its effect on CRC remains poorly understood. The present study investigated the effect of TSN on CRC SW480 cells and the AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling. Proliferation assay, flow cytometry and Hoechst 33342 nuclear staining demonstrated TSN dose-dependently inhibited cell viability and induced cell apoptosis as well as cell cycle arrest in S phase. Confocal laser scanning microscope showed  $\beta$ -catenin transferred to the outside of the nucleus in TSN-treated cells. Quantitative real-time PCR and western blot analysis found that TSN effectively modulated molecules related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling. Moreover, TSN administration significantly inhibited CRC growth in a mouse tumor xenograft model. In conclusion, our findings indicate that TSN inhibits growth and induces apoptosis in CRC cells through suppression of AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, suggesting that TSN may have potential for use in CRC treatment.

## Introduction

Colorectal cancer (CRC), a malignant disease with high morbidity and mortality, ranks the third most commonly diagnosed cancer in the world and accounts for 492,000 related

deaths annually (1,2). As a well-studied cancer, CRC is now recognized as a complex disease resulting from the accumulation of genetic and epigenetic alterations (3). Treatment strategies for CRC include surgery, chemotherapy and radiotherapy which have shown great improvement (4). However, the prognosis for CRC is unsatisfactory as recurrence and metastasis frequently occur. Application of chemotherapy to CRC is restricted due to high incidence of severe side-effects and drug resistance. Therefore, the research and development of new effective chemotherapeutic agents for CRC is urgently needed.

$\beta$ -catenin, the crucial molecule in Wnt/ $\beta$ -catenin pathway, promotes the transcription of several oncogenic target genes related to cancer progression (5).  $\beta$ -catenin has been found to drive cancer development and be active in 80% of CRC (6-9). It can be induced by phosphorylation and proteasome-mediated degradation by glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), which acts as a negative regulator of Wnt/ $\beta$ -catenin signaling pathway and is implicated in governing cancer cell proliferation and apoptosis (10,11). Notably, phosphorylation of GSK3 $\beta$  at Ser<sup>9</sup> by phosphorylated AKT (p-AKT) induces GSK3 $\beta$  inactivation and inhibits its ability to promote the degradation of  $\beta$ -catenin (12,13). As a result, AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling pathway has been indicated as an important therapeutic target for drug design to inhibit growth and metastasis of cancer cells.

Toosendanin (TSN), a triterpenoid as shown in Fig. 1, is a colorless and acicular crystal extracted from the bark or fruits of *Melia toosendan* Sieb et Zucc, which mainly grows in China and India and exhibits analgesic, insecticidal and anti-inflammatory activities (14). TSN is shown to possess antitumour effects on various human cancer cells *in vitro*, with IC<sub>50</sub> values ranging from 5.4 to 900 nM (15-17). *In vivo* experiments have shown that TSN exerts strongly suppressive effects on hepatocellular carcinoma in BALB/C mice (14). In addition, TSN induces apoptosis in several types of cancer cells by regulation of the mitochondrial pathway (14,15). However, sophisticated signaling pathways involved in TSN regulation of cancer cells including CRC have not been completely elucidated. Thus, the present study was performed

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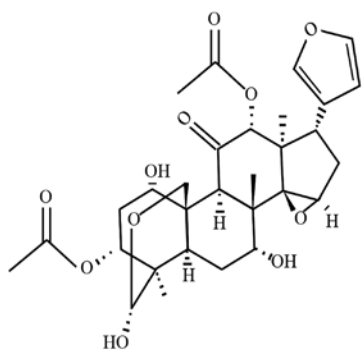


Figure 1. The chemical structure of toosendanin ( $C_{30}H_{38}O_{11}$ , molecular weight = 574.63).

to investigate the effect of TSN on CRC cells and related molecular mechanism.

### Materials and methods

**Chemicals and antibodies.** TSN (purity  $\geq 99\%$ ) was purchased from Xi'an Insecticide Biological Projected Co., Ltd. (Xi'an, China) and dissolved in DMSO; pancreatin and 3-(4,5)-Dimethylthiazol(-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from BioSharp (Hefei, China); RPMI-1640 medium and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY, USA); Annexin-V/propidium iodide (PI) apoptosis detection kit was from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China); TRIzol reagents and Power SYBR-Green PCR Master Mix were obtained from Invitrogen (Carlsbad, CA, USA); Primescript reverse transcription reagent kit with gDNA Eraser was from Takara (Dalian, China); monoclonal mouse  $\beta$ -actin antibody was from Sigma Chemical Co. (St. Louis, MO, USA); antibodies against Bcl-2, GSK-3 $\beta$ , pro-caspase-3, pro-PARP, vascular endothelial growth factor-A (VEGFA) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Antibodies against Bax, AKT, p-AKT Ser<sup>473</sup>, p-GSK-3 $\beta$  Ser<sup>9</sup>,  $\beta$ -catenin were from Cell Signaling Technology (Beverly, MA, USA).

**Cell line and cell culture.** Human CRC cell line SW480 was obtained from the Type Culture Collection, Chinese Academy of Sciences (Shanghai, China), cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) at a 37°C humidified atmosphere containing 5% CO<sub>2</sub>.

**Cell proliferation assay.** MTT assay was used to analyze cell proliferation. Briefly, cells in the logarithmic growth phase were plated in 96-well culture plates at a density of 5x10<sup>3</sup> cells/well. After adhering to the plate surface, cells were treated with TSN (0.5, 0.25, 0.125, 0.063 and 0.031  $\mu$ M) for 24 and 48 h, followed by 20  $\mu$ l of MTT (5 mg/ml) incubation for further 4 h. The formazan crystals formed were dissolved by the addition of DMSO and the optical densities (ODs) were measured by ELx800 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at 490 nm. The cell growth inhibition rate was calculated using the following formula: 1 - OD (experiment)/OD (control).

**Apoptosis assay.** Annexin V/PI apoptosis detection kit was used to assess induction of apoptosis. Briefly, cells (1x10<sup>6</sup>) treated with or without TSN (0.5 and 0.125  $\mu$ M) for 48 h, together with 50  $\mu$ g/ml 5-fluorouracil (5-FU) for 12 h as a positive control were harvested and suspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). Approximately 5  $\mu$ l Annexin V and 5  $\mu$ l PI were then added to the solution, afterwards the cells were gently vortexed and incubated for 15 min at room temperature in the dark for analysis by fluorescent activated cell sorting (FACS) on a flow cytometer.

**Cell cycle distribution analysis.** After incubation in serum-free medium for 24 h, cells were treated with TSN at different concentrations for another 24 h. The cells were then trypsinized, washed with PBS and fixed with 75% cold ethanol overnight at 4°C. After PBS washing, the fixed cells were stained with 50  $\mu$ g/ml PI in the presence of RNase for 30 min at 4°C in the dark. The stained cells were analyzed by flow cytometry. The DNA content in cells could be read according to PI fluorescence.

**Hoechst 33342 nuclear staining.** The trypsinized cells were plated onto coverslips in 6-well plates at a density of 1.5x10<sup>5</sup> cells/well. After incubation for 24 h, the cells were treated with 0.125 or 0.5  $\mu$ M TSN for 48 h. The cells were then washed with PBS and incubated with Hoechst 33342 for 30 min in CO<sub>2</sub> incubator. Following rinsing three times in PBS, the cells were examined for nuclear changes via fluorescent microscope (normal nuclei was identified as non-condensed chromatin dispersed over the entire nucleus and apoptotic nuclei was identified as condensed chromatin, contiguous with the nuclear membrane and/or fragmented nuclei).

**Analysis using confocal laser scanning microscopy.** After incubation on coverslips in 6-well plates at a density of 1.5x10<sup>5</sup> cells/well for 24 h, the cells were treated with 0.5  $\mu$ M TSN for 48 h. After PBS washing, the cells were fixed with pre-colling acetone for 15 min, permeabilized by 0.5% Triton X-100 for 10 min and blocked with PBS containing 5% BSA. Afterwards, the cells were incubated with primary antibodies overnight at 4°C. After washing three times with PBS containing 5% BSA, incubated with secondary antibodies for 1 h and mounted by mounting liquid containing DAPI, the cells were photographed using confocal laser scanning microscopy.

**Total RNA extraction and real-time PCR (RT-PCR).** Total RNA was isolated using the TRIzol reagent following the manufacturer's instructions. Briefly, after treated with 0.125 and 0.5  $\mu$ M TSN or 50  $\mu$ g/ml 5-Fu for 48 h, the cells were resuspended in 1 ml of TRIzol. The suspension was extracted by 0.2 ml of chloroform, and after centrifugation mixed with 0.5 ml of isopropyl alcohol, and the resulting pellet was washed with 0.7 ml of 75% ethanol and finally resuspended in 50  $\mu$ l RNase-free water. All total RNA samples were kept at -80°C until use. Reverse-transcription was carried out using M-MLV and cDNA amplification was carried out using SYBR-Green Master Mix kit according to the manufacturer's protocol. Human  $\beta$ -actin expression was used as internal control. Each sample was tested in triplicate with the

Table I. Sequences of the primers used in the real-time PCR amplifications.

Gene primer	Sequence (5'-3')	Length of PCR product (bp)
Bax	Forward: TTTGCTTCAGGGTTTCATCC Reverse: GCCACTCGGAAAAAGACCTC	213
Bak	Forward: ACGTGTCAGAAGCCTCCAAG Reverse: TGAGAGCCTTCACCTGTAGTG	110
Bcl-2	Forward: TCGCCCTGTGGATGACTGAG Reverse: CAGAGTCTTCAGAGACAGCCAGGA	143
Bcl-xL	Forward: ATGAACTCTTCCGGGATGG Reverse: TGGATCCAAGGCTCTAGGTG	166
Survivin	Forward: TTCTCAAGGACCACCGCATC Reverse: GCCAAGTCTGGCTCGTTCTC	127
ACTB	Forward: GGCCAACCGCGAGAAGAT Reverse: CGTCACCGGAGTCCATCA	134
$\beta$ -catenin	Forward: GGCCATATCCACCAGAGTGAA Reverse: GCCAATGGCTTGGAATGAGA	119
GSK-3 $\beta$	Forward: GACTAAGGTCTTCCGACCCC Reverse: AAGAGTGCAGGTGTGTCTCG	177
c-myc	Forward: CACCAGCAGCGACTCTGA Reverse: GATCCAGACTCTGACCTTTTGC	250
VEGFA	Forward: CCCTGATGAGATCGAGTACATCTT Reverse: CTTGTCTTGCTCTATCTTTCTTTGGTCT	224
Cyclin D1	Forward: GAAGTTGCAAAGTCCTGGAGC Reverse: ATGGTTTCCACTTCGCAGCA	221
Cyclin D2	Forward: CCGCAGTGCTCCTACTTCAA Reverse: GCCAAGAAACGGTCCAGGTA	152
Cyclin D3	Forward: TGCACATGATTCCTGGCCT Reverse: CTGTAGCACAGAGGGCCAAA	107
COX-2	Forward: ATACGACTTGCAGTGAGCGT Reverse: GGGTGGGAACAGCAAGGATT	200

use of the QuantiTect SYBR-Green PCR kit (Qiagen, Hilden, Germany) for 40 cycles on the ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR primer sequences are shown in Table I. Each sample was tested in triplicate. Cycle threshold (Ct) values were obtained graphically for the target genes and  $\beta$ -actin.  $\Delta$ Ct = Ct (target genes) - Ct (endogenous reference gene).  $\Delta\Delta$ Ct =  $\Delta$ Ct (treated samples) -  $\Delta$ Ct (control samples). The relative fold change in gene expression were calculated using the  $2^{-\Delta\Delta$ Ct} method.

**Western blot analysis.** Cells treated with TSN or 50  $\mu$ g/ml 5-Fu for 48 h were harvested with a cell scraper. Proteins were extracted with RIPA buffer containing protease inhibitor cocktail and protein concentrations were determined using Bradford assay. Equal amounts of protein (20  $\mu$ g) from each sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were then blocked with 5% skim milk for at least 30 min. The primary antibodies were diluted according

to the manufacturer's instructions. Afterwards, the membranes were incubated with appropriate primary antibodies overnight at 4°C, washed three times with PBST and incubated with horseradish peroxidase-linked secondary antibodies at a dilution ratio of 1:1,000 for 1 h at room temperature. Then the immunoreactive bands were detected using an ECL detection kit (Millipore).  $\beta$ -actin was used as a loading control. Three separate experiments were performed for each sample.

**Animals and in vivo tumor xenograft studies.** Male BALB/c/nu/nu nude mice weighing 18-22 g were obtained from Shanghai Experimental Animal Center of the Chinese Academy of Sciences. All animal experiments were approved by the Animal Ethics and Research Committee of Shanghai Jiaotong University. SW480 cells ( $5 \times 10^7$ ) were injected subcutaneously into the right flank of mice (n=30). When the tumor diameter reached  $\sim$ 5 mm, mice were randomly divided into 3 groups: control (mice receiving PBS; n=10), low-dose TSN (0.15 mg/kg; n=10), and high-dose TSN (0.30 mg/kg; n=10). The PBS and TSN were intraperitoneally given once daily for

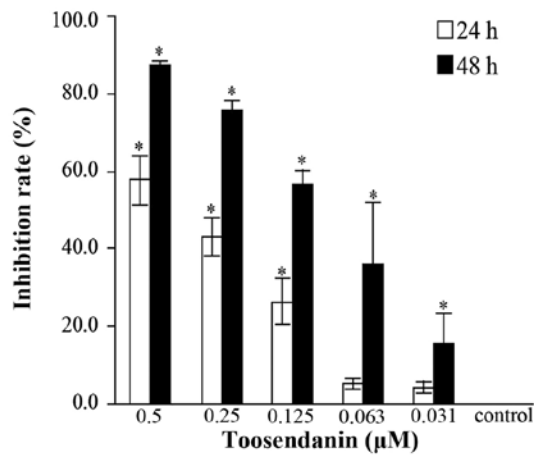


Figure 2. The effect of TSN on proliferation of SW480 cells. MTT assay showed that TSN exhibited inhibitory effect on proliferation of SW480 cells in dose- and time-dependent manner. \* $P < 0.05$ .

14 days. When the treatment began, the mean tumor volumes were calculated every 3 days with a caliper, using the formula volume = (length  $\times$  width<sup>2</sup>)/2. The mice were sacrificed 24 h after the final dose and tumors were resected aseptically for weight and volume calculation.

**Statistical analysis.** The data are expressed as the mean  $\pm$  standard deviation (SD). The statistical differences of experimental data between the groups were determined by SPSS 17.0 software using one-way ANOVA test. Significance was defined as  $P < 0.05$  or  $P < 0.01$ .

## Results

**Effects of TSN on cell proliferation.** In order to examine the effects of TSN on CRC SW480 cell proliferation *in vitro*, MTT

assay was used. As a result, it was found that cell proliferation of SW480 cells was significantly suppressed when treated by TSN and the inhibition rates were both dose- and time-dependent (Fig. 2). The  $IC_{50}$  value was  $0.3493 \mu M$  for 24 h and  $0.1059 \mu M$  for 48 h.

**TSN induces cell apoptosis.** Analysis of Annexin V/PI double staining cells by flow cytometry showed apoptosis of SW480 cells by TSN. After treatment for 48 h, the early apoptosis (lower right quadrant) rates were 21.80 and 35.67% for TSN of 0.125 and  $0.5 \mu M$ , but 6.21 and 26.80% for negative control and positive control (5-Fu), respectively (Fig. 3A). Hoechst 33342 nuclear staining was used to explore the morphological alterations of cells after TSN (0.125 and  $0.5 \mu M$ ) treatment for 48 h. As indicated in Fig. 3B, the features of apoptosis including nuclear shrinkage, chromatin condensation and nucleolus fragmentation in TSN treated cells were investigated as compared with the control. Taken together, the above results demonstrated that TSN was able to effectively induce apoptosis of SW480 cell in dose-dependent manner.

**TSN induced cycle arrest.** The cell cycle of SW480 cells treated with TSN was analyzed by flow cytometric analysis. The proportion of cells in the S phase increased while that in the G0/G1 phase decreased in a dose-dependent manner resulting from treatment with TSN (0.125 and  $0.5 \mu M$ ) (Fig. 4). The results indicated that inhibitory effect on SW480 cell proliferation by TSN is possibly mediated by blocking cellular progress through the S phase.

**Effects of TSN on the nucleus translocation of  $\beta$ -catenin in SW480 cell.** Confocal laser scanning microscope was used to observe the nucleus translocation of  $\beta$ -catenin after TSN treatment in SW480 cells. In the negative control,  $\beta$ -catenin was accumulated in the nucleus, whereas  $\beta$ -catenin transferred to

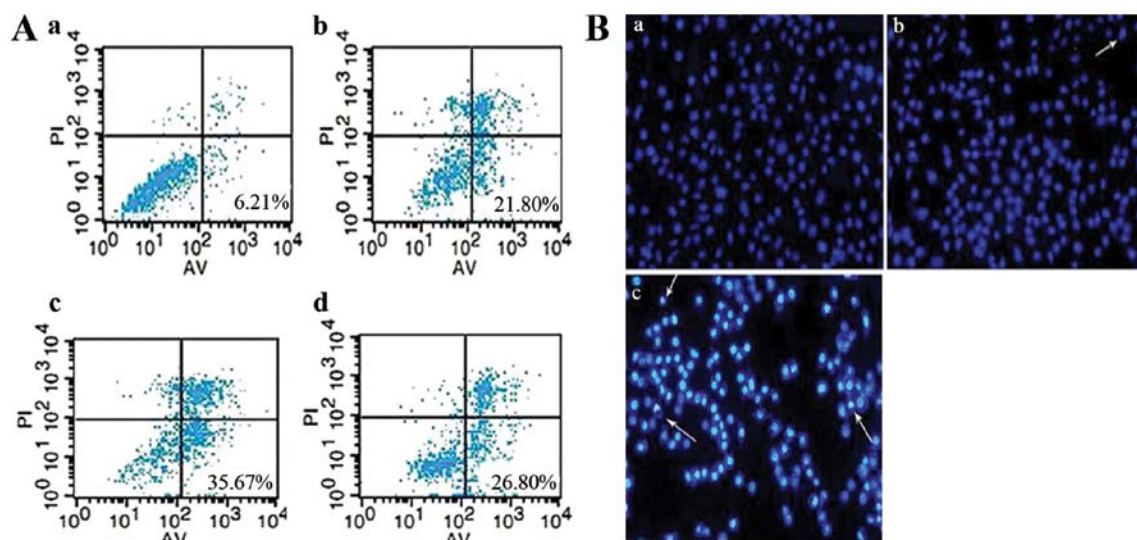


Figure 3. (A) Apoptosis and (B) apoptotic morphological changes in SW480 cells after TSN treatment. a, control; b,  $0.125 \mu M$  TSN; c,  $0.5 \mu M$  TSN; d,  $50 \mu g/ml$  5-Fu. Lower left (LL) quadrants, living cells (AV negative/PI negative); lower right (LR) quadrants, early apoptotic cells (AV positive/PI negative); upper right (UR) quadrants, late apoptotic cells (AV positive/PI positive); upper left (UL) quadrants, necrotic cells (AV negative/PI positive). The numbers represent the percentage of the cells in the sum of LR quadrants. For apoptotic morphological changes, apoptotic cells were detected by Hoechst 33342 staining and measured by fluorescence microscopy (magnification,  $\times 200$ ).

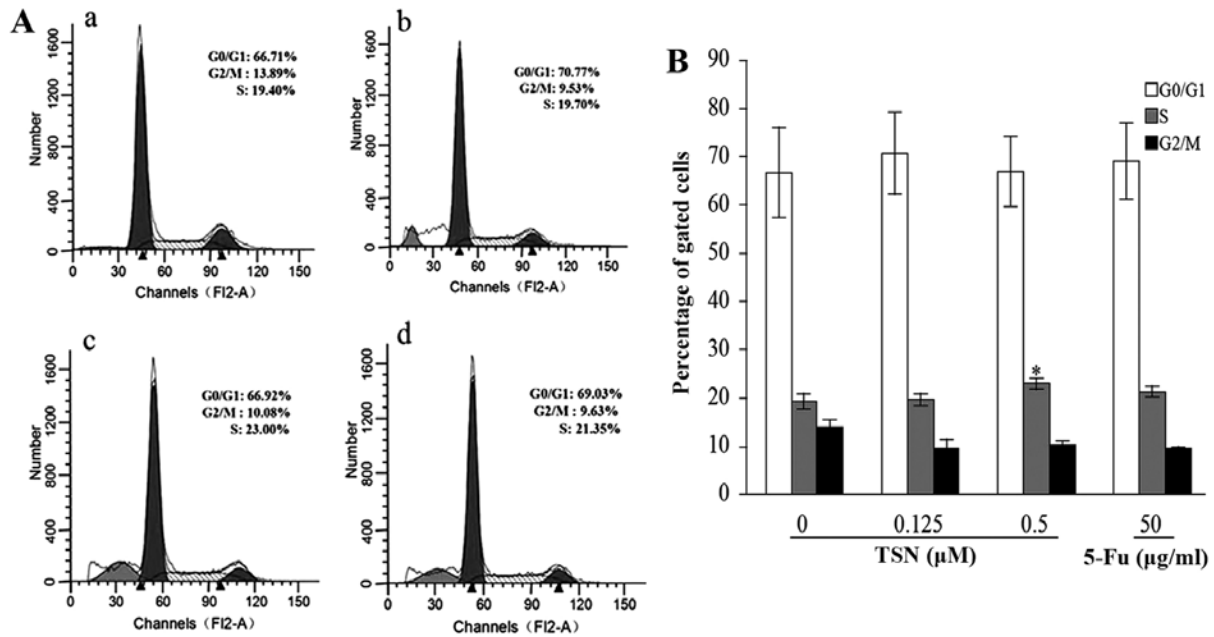


Figure 4. Cell cycle profile of SW480 cells after 24-h TSN treatment. (A) The cell cycle profile were analyzed with flow cytometry and representative results are shown. a, control; b, 0.125  $\mu$ M TSN; c, 0.5  $\mu$ M TSN; d, 50  $\mu$ g/ml 5-Fu. (B) The distribution of the cell cycle was calculated and shown by columns representing means  $\pm$  SD of three independent experiments. \* $P < 0.05$  compared with control.

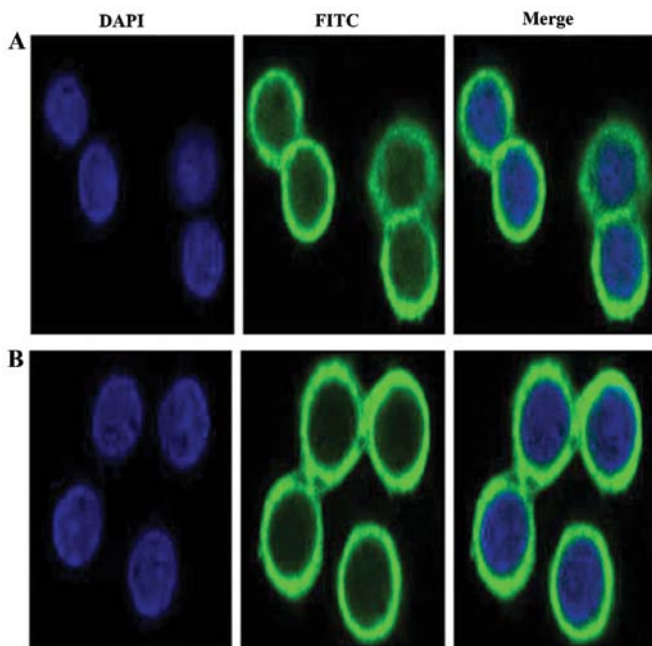


Figure 5. Effects of TSN on the nucleus translocation of  $\beta$ -catenin in SW480 cells. Confocal laser scanning microscope was used to observe the nucleus translocation of  $\beta$ -catenin after TSN treatment on SW480 cells for 48 h. (A) control; (B) 0.5  $\mu$ M TSN.

the outside of the nucleus when SW480 cells were treated by TSN of 0.5  $\mu$ M for 48 h (Fig. 5).

**Effects of TSN on the mRNA expression of genes related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway.** RT-PCR was used to investigate the effects of TSN on the mRNA changes of molecules related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. TSN could significantly increase the mRNA levels of

Bax, Bak and GSK-3 $\beta$  while decrease those of Bcl-2, Bcl-xL, Survivin, cyclin D1, cyclin D2, cyclin D3,  $\beta$ -catenin, VEGFA, c-myc and COX-2 in a dose-dependent manner (Fig. 6A). The results demonstrated that the apoptosis-induction effects on SW480 cells by TSN might be associated with regulation of mRNA expressions of genes in Bcl-2 family and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway.

**Effects of TSN on the protein expression related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway.** Western blot analysis was performed to analyze the changes of proteins related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. After treatment with TSN for 48 h, protein levels of Bcl-2, pro-caspase-3 and Pro-PARP decreased while that of Bax increased in a dose-dependent manner. Besides, a significant decrease of AKT, P-AKT Ser<sup>473</sup>, P-GSK-3 $\beta$  Ser<sup>9</sup>,  $\beta$ -catenin and VEGFA and increase of GSK-3 $\beta$  was observed after TSN treatment (Fig. 6B). These results indicated that TSN induced the apoptosis of SW480 cells through regulating Bcl-2 family and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway as well as activating the caspase-cascade response.

**Effects of TSN administration on xenograft tumor growth.** The effects of TSN on xenograft tumor growth were investigated *in vivo*. During the whole tumor growth period, the tumor volume was measured. Tumors of mice in low or high-dose TSN group grew more slowly compared with those in control group (Fig. 7A and C). The average weight and volume of the finally resected tumors in the TSN-treated groups were significantly lower than those of the control group (Fig. 7B and D). Nevertheless, a significant difference in tumor weight and volume was not found between the low- and high-dose TSN groups. This result strongly suggested that TSN administration inhibited CRC growth *in vivo*.

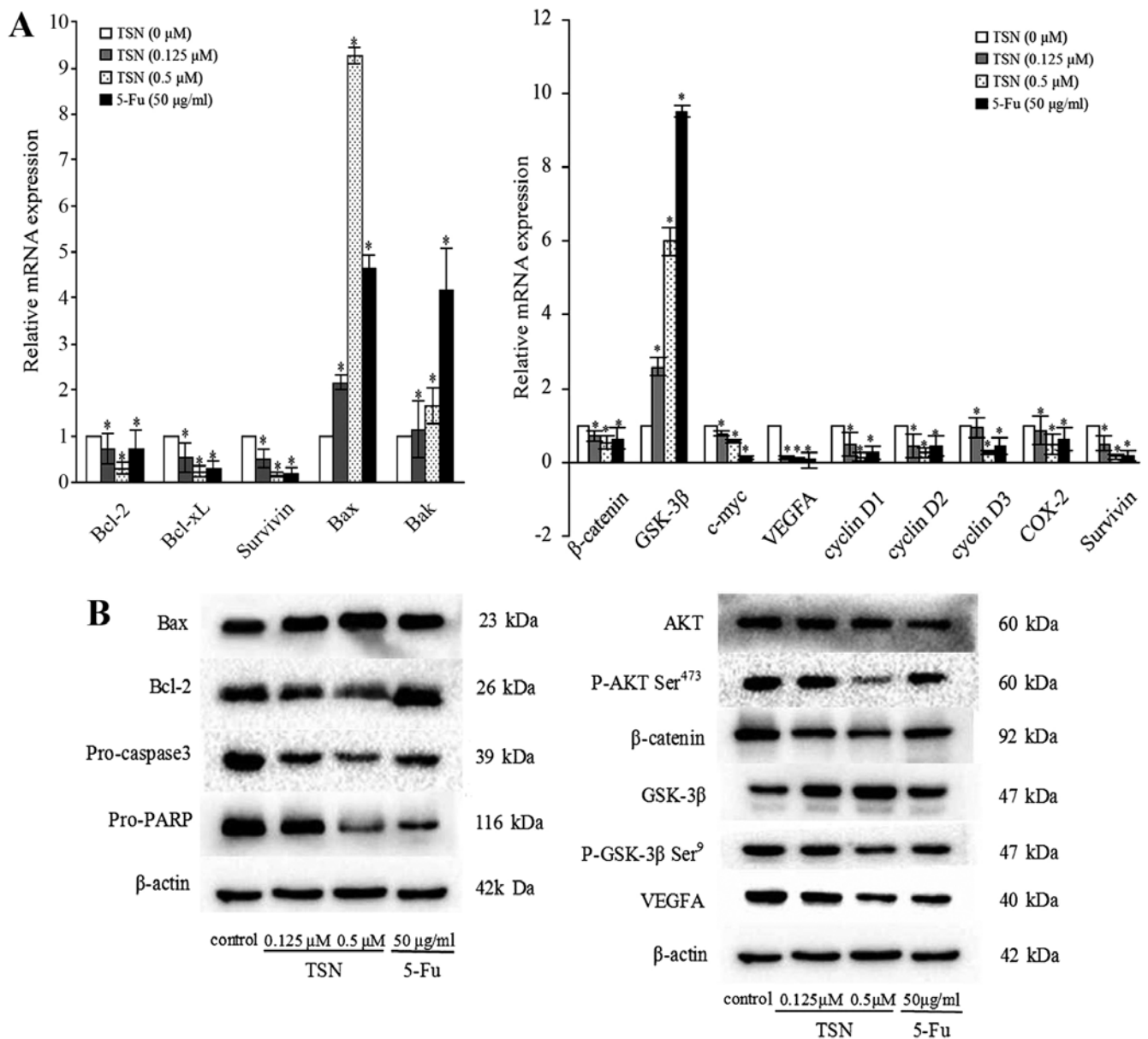


Figure 6. Effects of TSN on the mRNA and protein expression of genes related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. (A) RT-PCR analysis of genes related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling. After SW480 cells were treated for 48 h with or without TSN (0.125 and 0.5  $\mu$ M), the mRNA levels of genes (Bcl-2, Bcl-xL, Bax, Bak,  $\beta$ -catenin, GSK-3 $\beta$ , c-myc, VEGFA, cyclin D1, cyclin D2, cyclin D3, COX-2 and survivin) were determined by RT-PCR.  $\beta$ -actin was taken as an internal control and 5-Fu (50  $\mu$ g/ml) as a positive control. \* $P$ <0.05 compared with control. The data are representative of three independent experiments. (B) Western blot analysis of genes related to apoptosis and AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling. After SW480 cells were treated for 48 h with or without TSN (0.125 and 0.5  $\mu$ M), the protein levels of genes (Bax, Bcl-2, Pro-caspase-3, Pro-PARP, AKT, P-AKT Ser<sup>473</sup>,  $\beta$ -catenin, GSK-3 $\beta$ , P-GSK-3 $\beta$  Ser<sup>9</sup> and VEGFA) were determined by western blot analysis.  $\beta$ -actin was taken as an internal control and 5-Fu (50  $\mu$ g/ml) as a positive control.

## Discussion

Although chemotherapy is an important therapeutic strategy for CRC, the prognosis is still poor due to low response rates to most chemotherapeutic agents and severe toxicities. Natural plants and their effective components have been studied by pharmacologists and chemists for their diversity of chemical structure and promising therapeutic applications to cancer (18,19). Toosendanin is such a plant component which has significant inhibitory effects on various cancers including pheochromocytoma (15), hepatoma (14), leukemia (20), glioblastoma, neuroblastoma, prostate adenocarcinoma and lymphoma (21). Herein, we reported that TSN exhibited

suppressive activity of the viability of CRC SW480 cells in a time- and dose-dependent manner with IC<sub>50</sub> values of 0.349  $\mu$ M for 24 h and 0.1059  $\mu$ M for 48 h, respectively. The abnormal regulation of cell cycle is suggested to be the driving factor of tumor formation and regulators in cell cycle are often considered to be potential therapeutic targets (22). Cyclin D1, a recognized oncogene, can affect G1/S phase control point in the cell cycle. The overexpression of cyclin D1 results in disorder regulation of cell cycle and abnormal cell proliferation (23). Cyclin D2 and cyclin D3 act in dysregulating normal cell cycle and promoting the proliferation of cancer cells (24). Our results indicated that TSN suppressed proliferation of SW480 cell possibly by blocking the cell cycle through S phase.



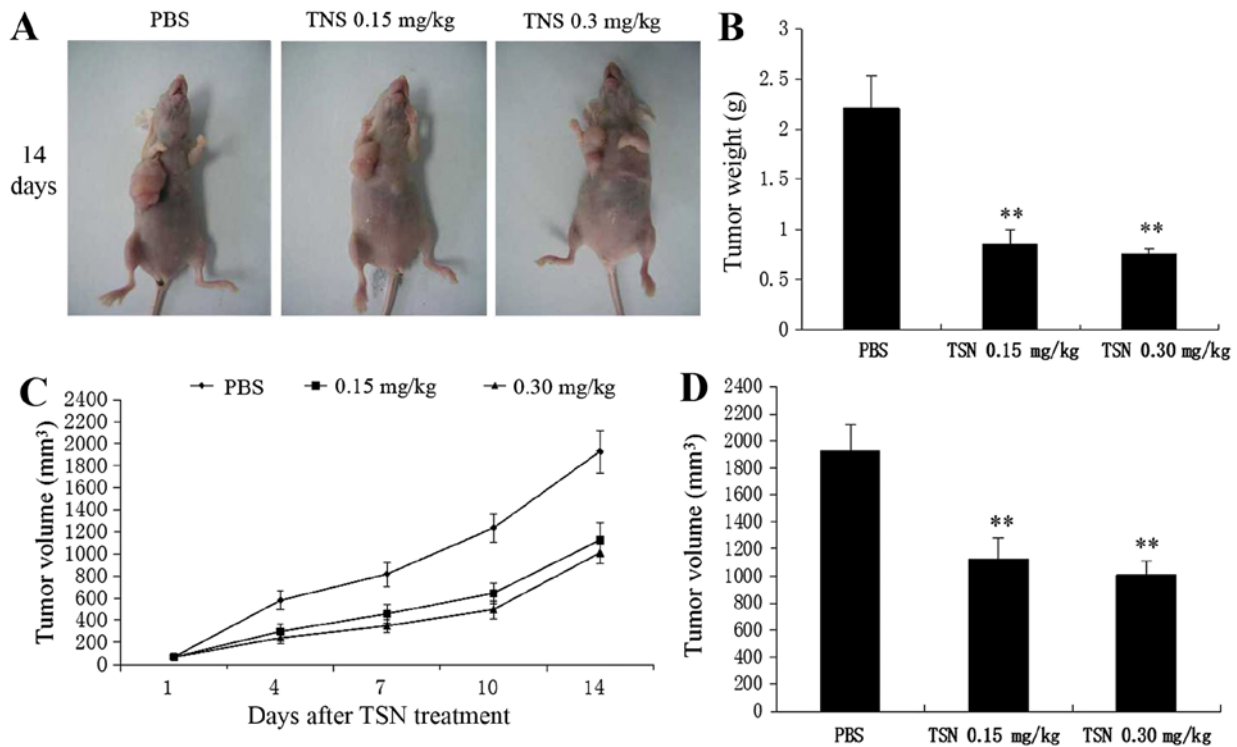


Figure 7. Inhibition of the growth of xenograft tumors derived from SW480 cells by TSN administration. (A) The image shows the difference of tumor volume between the TSN-treated groups and the control group on Day 14. (C) Tumor volume was measured after TSN treatment and the tumor volume curve is presented. (B and D) The differences of weight and volume of resected tumor between the TSN-treated groups and the control group were statistically significant (\*\* $P < 0.01$ ).

RT-PCR assay further showed that the molecular mechanism might be associated with downregulating the mRNA expressions of cyclin D1, cyclin D2 and cyclin D3.

Apoptosis, which differs from necrosis, is a pattern of programmed death. Lack of apoptosis is closely related to tumorigenesis and death of cancer cells occurs chiefly via apoptosis rather than necrosis (25). Apoptosis occurs in the phase in which cellular progress is blocked. The present study using flow cytometry and Hoechst 33342 nuclear staining showed that TSN treatment of 0.125 and 0.5  $\mu\text{M}$  for 48 h increased apoptosis rates of SW480 cells and revealed the typical apoptotic morphological alterations. All the above results indicated that induction of cell apoptosis and cell cycle arrest might contribute to the growth inhibition effects on CRC cells by TSN.

Caspases are a family of cysteine proteases which play a central part in the initiation and execution of apoptosis (14). Caspase-3, activated only by upstream initiator caspases, is an executioner caspase and induces apoptosis (26). PARP is one of the substrates of caspase-3 and plays a role in repairing DNA damage induced by anticancer agents or radiation. During apoptosis, caspase-3 restrains the activity of PARP by cleaving it into two fragments, p89 and p24 (27). Caspase-cascade is regulated by various factors, such as Bcl-2 family proteins involved in promoting (Bcl-2, Bcl-xL) or inhibiting (Bax and Bak) apoptosis (28). RT-PCR and western blot analysis in the present study revealed that the expressions of Bax and Bak increased while those of Pro-caspase-3, Pro-PARP, Bcl-2 and Bcl-xL decreased in TSN-treated CRC cells. Therefore, we hypothesized that TSN induced SW480 cell apoptosis through

activating the caspase-cascade and modulating Bcl-2 family molecules. Nevertheless, further research is needed to identify whether apoptosis-induction in SW480 cell by TSN depends on the extrinsic (death receptor), or the intrinsic (mitochondrial) pathway, or both.

A large body of evidence exists indicating that dysregulation of PI3K/AKT and Wnt signaling pathways plays important roles in the progression of CRC (29). The interplay between the two pathways through AKT-GSK-3 $\beta$ - $\beta$ -catenin axis is found to participate in vitality of cancer stem cells (30). In the axis,  $\beta$ -catenin enters the nucleus and binds with T cell factor/lymphoid enhancer factor (TCF/LEF)-1 proteins, followed by the transactivation of downstream target oncogenes such as cyclin D, surviving, VEGF, c-Myc and COX-2 (31-33). As a result,  $\beta$ -catenin serves as a powerful transcription factor that promotes cell proliferation in this way (34). Moreover, GSK-3 $\beta$  leads to phosphorylation and then proteasome-mediated degradation of  $\beta$ -catenin and activity of GSK-3 $\beta$  can be inhibited resulting from phosphorylation in serine-9 of GSK-3 $\beta$  by p-AKT (10,13). Thus, AKT phosphorylation of GSK-3 $\beta$  results in  $\beta$ -catenin stabilization and translocation to the nucleus. In the present study, we provide evidence that TSN treatment translocated  $\beta$ -catenin outside the nucleus and attenuated levels of AKT, p-AKT Ser<sup>473</sup>, p-GSK-3 $\beta$  Ser<sup>9</sup>,  $\beta$ -catenin as well as survivin, cyclin D, VEGFA, c-Myc and COX-2 which are  $\beta$ -catenin-activated genes. The expression of GSK-3 $\beta$  was upregulated by TSN. All the findings suggested that TSN inhibited the activity of  $\beta$ -catenin through suppressing p-AKT, activating GSK-3 $\beta$  and then inducing degradation of  $\beta$ -catenin. Importantly, mounting research has confirmed that down-

stream genes of  $\beta$ -catenin such as survivin, cyclin D, VEGFA, c-Myc and COX-2 are involved in promoting tumor growth and antiapoptosis (35,36). Bcl-2 activity can be regulated by  $\beta$ -catenin pathway in CRC progression (37). Therefore, the results in the present study suggested that the molecular mechanism of inhibiting proliferation and inducing apoptosis by TSN may lie on suppression of the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. *In vivo* study demonstrated that TSN administration significantly inhibited CRC growth in the mouse tumor xenograft model. In summary, our research might provide an experimental basis for TSN as a new chemotherapy drug against CRC.

In conclusion, our findings indicate that TSN inhibits growth and induces apoptosis in CRC cells through suppression of the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, suggesting that TSN may possess potential for use in CRC treatment.

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