ORIGINAL RESEARCH

Tetrandrine Reverses Paclitaxel Resistance in Human Ovarian Cancer via Inducing Apoptosis, Cell Cycle Arrest Through β-Catenin Pathway

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Background: Paclitaxel (PTX) resistance is a great obstacle for the treatment of ovarian cancer. A previous study indicated that tetrandrine (TET) could induce the apoptosis of ovarian cancer cells. This study aimed to explore the effect of TET in combination with PTX on PTX resistance in ovarian cancer cells.

Materials and Methods: CCK-8 assay, flow cytometry and wound healing assays were used to detect the proliferation, apoptosis and migration of PTX-resistant SKOV3 cells (SKOV3/PTX). The expressions of Bax, Bcl-2, cleaved caspase 3, β-catenin, c-Myc, cyclin D1 and p21 in SKOV3/PTX cells were detected with Western blot. In vivo animal study was performed finally.

Results: In this study, the inhibitory effects of PTX on the proliferation and migration of SKOV3/PTX cells were markedly enhanced by TET. In addition, PTX-induced apoptosis in SKOV3/PTX cells was significantly enhanced by the treatment of TET via upregulating the levels of Bax and cleaved caspase 3, and downregulating the expression of Bcl-2. Moreover, combination of TET and PTX obviously induced cell cycle arrest in SKOV3/PTX cells via increasing the level of p21 and decreasing the levels of c-Myc and Cyclin D1. Meanwhile, combination of TET with PTX significantly decreased the expression of β-catenin in SKOV3/PTX cells. In vivo experiments further confirmed that TET enhanced the anti-tumor effect of PTX in SKOV3/PTX xenograft model.

Conclusion: We found that TET could enhance the sensitivity of SKOV3/PTX cells to PTX via inhibiting the β-catenin/c-Myc/Cyclin D1 signaling pathway. Therefore, PTX combined with TET might be considered as a potential approach for the treatment of PTX-resistant ovarian cancer.

Keywords: ovarian cancer, paclitaxel, tetrandrine, apoptosis, cell cycle arrest

Introduction

Ovarian cancer is the fourth most common cancer among women, especially in older women.[1] It often occurs in peri- and post-menopausal women.[2] Age is the main cause, more than 48% of ovarian cancers occur in women over the age of 65 years.[3] According to the International Federation of Gynecology and Obstetrics (FIGO) system, ovarian cancer is divided into four stages: range from stage I to IV.[4] Stage I and stage II tumors are called early-stage cancer which spread is limited to the pelvic cavity, and stage III and stage IV tumors that spread outside of the pelvic cavity are called advanced or metastatic cancer.[4,5] Currently, chemotherapy and surgery are the main therapeutic methods for the treatment of ovarian cancer.[6,7]

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Previous study found that chemotherapy could decrease the tumor recurrence rate and metastasis rate, whereas drug resistance occurred frequently after long-term chemotherapy.[8]

Paclitaxel (PTX) is a first-line chemotherapy drug for the treatment of ovarian cancer.[9] However, PTX resistance is one of the key factors affecting the effect of chemotherapy, which markedly limit the clinical usage of PTX.[10,11] Therefore, searching new agents to overcome cancer chemoresistance is a novel effective therapy for the treatment of ovarian cancer.

Tetrandrine (TET) is one of the most effective component of the dried root of traditional Chinese medicine *Stephania tetrandra* S.[12] Recent studies have revealed that TET exhibited beneficial effects in a number of types of human cancers including lung cancer, breast cancer, colon cancer and cervical cancer.[13–16] Li et al indicated that TET administration induced the growth of colon carcinoma cells via inducing apoptosis and cell cycle arrest.[17] Meanwhile, Zhang et al indicated that TET could reverse cisplatin resistance in ovarian cancer cells by inducing apoptosis.[18] However, the effect of TET in PTX resistance of ovarian cancer remains unclear. Therefore, we aimed to explore the anti-tumor effect of combination of TET and PTX on PTX-resistant human ovarian cancer cells.

Materials and Methods
Cell Culture
The human ovary cancer cell line SKOV-3 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human ovary cancer cell line A2780 was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PTX-resistant SKOV-3 cell line (SKOV3/PTX) were established by long-term exposure of SKOV-3 cells to a stepwise gradually concentration of PTX over 7 months. Cells were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2.

CCK-8 Assay
Cell viability was determined using a Cell Counting Kit 8 (CCK-8, Abcam Cambridge, MA, USA). SKOV-3, A2780 or SKOV3/PTX cells were seeded into 96-well plates at a density of 5×10^3 cells/well and incubated at 37°C overnight. After that, cells were treated with different concentrations of PTX for 72 h. Subsequently, 10 μL of CCK8 reagent was added into each well and then incubated for another 2 h. Finally, the optical density (OD) value of cells was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Immunofluorescence Staining Assay
SKOV3/PTX cells (5×10^3 cells/well) were seeded onto 96-well plates and incubated at 37°C overnight. Cells were fixed in 3.7% paraformaldehyde for 15 min at room temperature, and then permeabilized with 0.2% Triton X-100 for 5 min. After that, the cells were washed twice with PBS and then incubated with the primary antibody anti-Ki67 (1:1000, Abcam) at 4°C overnight. Later on, cells were incubated with a fluorescence-conjugated secondary antibody (1:2000, Abcam) at 37°C for 1 h. Nuclei was counterstained with DAPI for 10 min. A fluorescence microscope (Olympus, Tokyo, Japan) was applied to detect the fluorescence signal.

Apoptosis Assay by Flow Cytometry
The Annexin V-FITC apoptosis detection kit was used to measure cell apoptosis (Thermo Fisher Scientific). SKOV3/PTX cells were incubated with PTX or/and TET for 72 h at 37°C. After that, cells were collected and washed twice with PBS, and then stained with 5 μL annexin V-FITC and 5 μL propidium iodide for 30 min in the dark. Finally, apoptosis cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA).

JC-1 Staining
Mitochondrial membrane potential (MMP) was assessed by cationic dye JC-1 using the MMP assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, SKOV3/PTX cells were collected and washed twice with PBS. Later on, cells were incubated with 10 mg/mL JC-1 reagent at 37°C for 20 min in the dark. After that, cells were resuspended in PBS for analysis by the flow cytometer, as previously described.[19,20]

Western Blot
BCA protein Assay kit (Beyotime Institute of Biotechnology) was applied to determine the concentration of protein. Proteins from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto the polyvinylidene difluoride membrane (PVDF, Thermo Fisher Scientific). After that, the membrane was blocked in 5% nonfat milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room

temperature. Then, the membrane was incubated in primary antibodies at 4°C overnight, including anti-Cyclin D1 (1:1000, Abcam), anti-p21 (1:1000, Abcam), anti-Bax (1:1000, Abcam), anti-Bcl-2 (1:1000, Abcam), anti-cleaved caspase 3 (1:1000, Abcam), anti-c-Myc (1:1000, Abcam), and anti-β-actin (1:1000, Abcam). After washing with TBST, membranes were incubated with goat anti-rabbit secondary antibodies (1:5000, Abcam) for 1 h at the room temperature. Then, the protein bands were visualized using an ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific). β-actin was acted as the internal control.

Wound Healing Assay

SKOV3/PTX cells were seeded into six wells culture plate overnight at 37°C. The cellular monolayer was wounded with a sterile micropipette tip. Then, cells were incubated with PTX or/and TET for 24 h at 37°C. Images of the scratched wounds were observed at 0 h and 24 h using the fluorescence microscope (Olympus CX23 Tokyo, Japan). The migrated areas were determined using ImageJ software (version 1.52).

Cell Cycle

SKOV3/PTX cells were incubated with PTX or/and TET for 24 h at 37°C. After that, cells were fixed in ice-cold 70% ethanol at 4°C overnight. Then, cells were resuspended and washed twice with PBS. Later on, cells were incubated with staining buffer containing 50 μg/mL PI in the dark at 4°C for 30 min. Finally, cell-cycle distribution was analyzed by a flow cytometer (BD Biosciences).

Animal Study

BALB/c nude female mice (n = 12, 6–8 weeks old) were obtained from Animal Center of Chinese Academy of Sciences (Shanghai, China). All mice were maintained following the guidelines of the Institutional Animal Care and Use Committee. These animal experiments were performed approved by the Ethics Committees of Shengjing Hospital Affiliated to China Medical University. SKOV3/PTX cells (5×10^6 cells) were transplanted subcutaneously into the right flank in each animal. When the tumor volume reached about 180 mm^3, the mice were randomized into four groups: vehicle, PTX_10 mg/kg, TET_50 mg/kg and PTX (10 mg/kg) + TET (50 mg/kg) groups. TET was administered via intraperitoneal (i.p.) injection daily. PTX was injected intravenously once every three days. Tumor volume was monitored weekly and analyzed using the formula V = (length x width^2)/2. After 3 weeks, mice were sacrificed and tumor tissues were weighted.

Statistical Analysis

All data were repeated in triplicate. All statistical analyses were performed using GraphPad Prism software (version 7.0, La Jolla, CA, USA). One-way analysis of variance (ANOVA) and Tukey's tests were carried out for multiple group comparisons. All data were presented as mean ± standard error (S. D.). A P-value < 0.05 was considered as a statistically significant.

Results

TET Enhanced the Anti-Proliferation Effect of PTX in SKOV3/PTX Cells

First, we established a PTX-resistant SKOV-3 cell line (SKOV3/PTX). Cell proliferation assay indicated that SKOV3/PTX cells were resistant to PTX after gradient treatment compared with SKOV3 parental cells (Figure 1A). To evaluate the effects of TET on the viability of PTX-resistance SKOV3 cells, CCK-8 assay was used. As shown in Figure 1B, TET (2, 4 or 8 μM) significantly inhibited the proliferation of SKOV3/PTX cells. In addition, TET (2, 4 or 8 μM) notably enhanced the anti-proliferation effect of PTX (1 μM) on SKOV3/PTX cells (Figure 1C). Moreover, combination of 1 μM PTX with 2 μM TET induced about 50% growth inhibition in SKOV3/PTX cells (Figure 1C). Therefore, 2 μM TET was utilized in the following experiments.

Next, we found PTX significantly decreased the number of Ki67-positive SKOV3/PTX cells compared to control group. As expected, the Ki67-positive cells were further obviously reduced in the presence of TET, compared with PTX treatment group (Figure 1D and E). Furthermore, as shown in Table 1, IC$_{50}$ value of TET (alone treatment) was 8.48 μM in SKOV3/PTX cells. However, when TET was combined with PTX (1 μM), the IC$_{50}$ value of TET was decreased to 1.5 μM. In addition, the CI value of TET combined with PTX in SKOV3/PTX cells was less than 0.3, which indicated the strong synergism effect (Table 1). These data indicated that combination of TET with PTX had synergistic effect against SKOV3/PTX cells.

In addition, as shown in Supplementary Figure 1A–D, TET or PTX significantly inhibited the proliferation of parental SKOV3 and A2780 cells. As expected, combination treatment of TET and PTX markedly suppressed the proliferation of SKOV3 and A2780 cells, compared with PTX treatment group (Supplementary Figure 1A–D). These results indicated that TET could enhance the anti-proliferation effect of PTX in ovarian cancer cells.
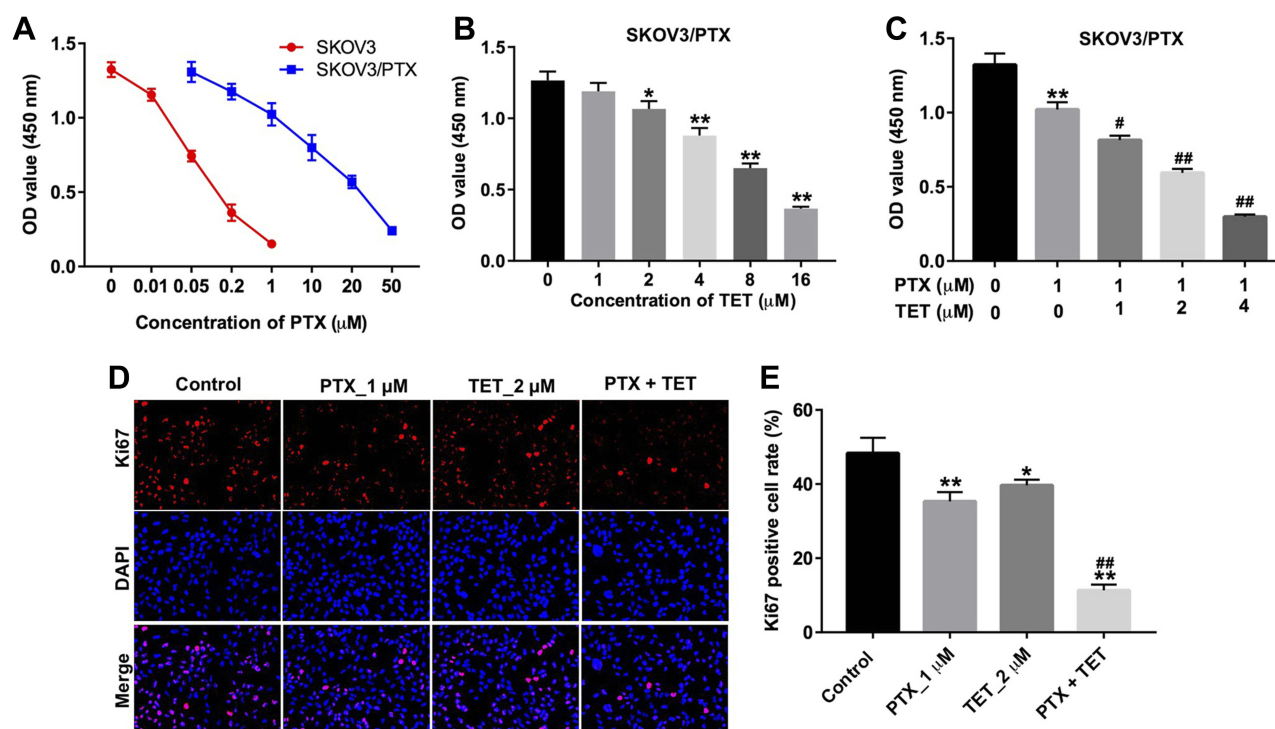
Figure 1 TET enhanced the anti-proliferation effect of PTX in SKOV3/PTX cells. (**A**) CCK-8 assay was used to detect the cell viability. SKOV3 cells were treated with 0, 0.01, 0.05, 0.2, or 1 μM PTX for 72 h. SKOV3/PTX cells were treated with 0, 0.05, 0.2, 1, 10, 20 or 50 μM PTX for 72 h. (**B**) SKOV3/PTX cells were treated with 0, 1, 2, 4 or 8 μM TET for 72 h. (**C**) SKOV3/PTX cells were treated with PTX (1 μM) and TET (0, 1, 2 or 4 μM) for 72 h. (**D**, **E**) SKOV3/PTX cells were treated with PTX (1 μM) or/and 2 μM TET for 72 h. Quantification of Ki67 expression by immunofluorescence in SKOV3/PTX cells. *P<0.05, **P<0.01 compared with control group; #P<0.05, ##P<0.01 compared with PTX_1 μM group.

TET Enhanced the Pro-Apoptotic Effect of PTX in SKOV3/PTX Cells

Flow cytometric assay was used to evaluate the apoptosis in SKOV3/PTX cells. As illustrated in Figure 2A and B, PTX notably induced the apoptosis of SKOV3/PTX cells. As expected, the apoptotic cells were further increased in the presence of TET, compared with PTX treatment group. In addition, combination of PTX with TET markedly decreased the MMP in SKOV3/PTX cells, compared with PTX treatment group (Figure 2C). Moreover, PTX significantly increased the levels of Bax and cleaved caspase 3, but decreased the expression of Bcl-2 in SKOV3/PTX cells. As expected, the levels of Bax and active caspase 3 in cells were further increased in the presence of TET, while the expression of Bcl-2 was further decreased, compared with PTX treatment

Table 1 Evaluation of Combination of PTX with TET in Ovarian Cancer (72 h Treatment)

Combination Strategy	IC 50 Value of TET	CI Values
TET	IC50 = 8.48 μM	–
TET + 1 μM PTX	IC50 = 1.50 μM	0.25

group (Figure 2D–G). These results indicated that TET could enhance the pro-apoptotic effect of PTX in SKOV3/PTX cells.

TET Enhanced the Anti-Migratory Effect of PTX in SKOV3/PTX Cells

Next, the effect of TET and PTX on the migration of SKOV3/PTX cells was analyzed by wound-healing assay. As indicated in Figure 3A and B, PTX or TET obviously suppressed the migration ability of SKOV3/PTX cells. Meanwhile, combination of PTX with TET further inhibited the migration ability of SKOV3/PTX cells, compared with PTX alone treatment groups (Figure 3A and B). These results indicated that TET could enhance the anti-migratory effect of PTX in SKOV3/PTX cells.
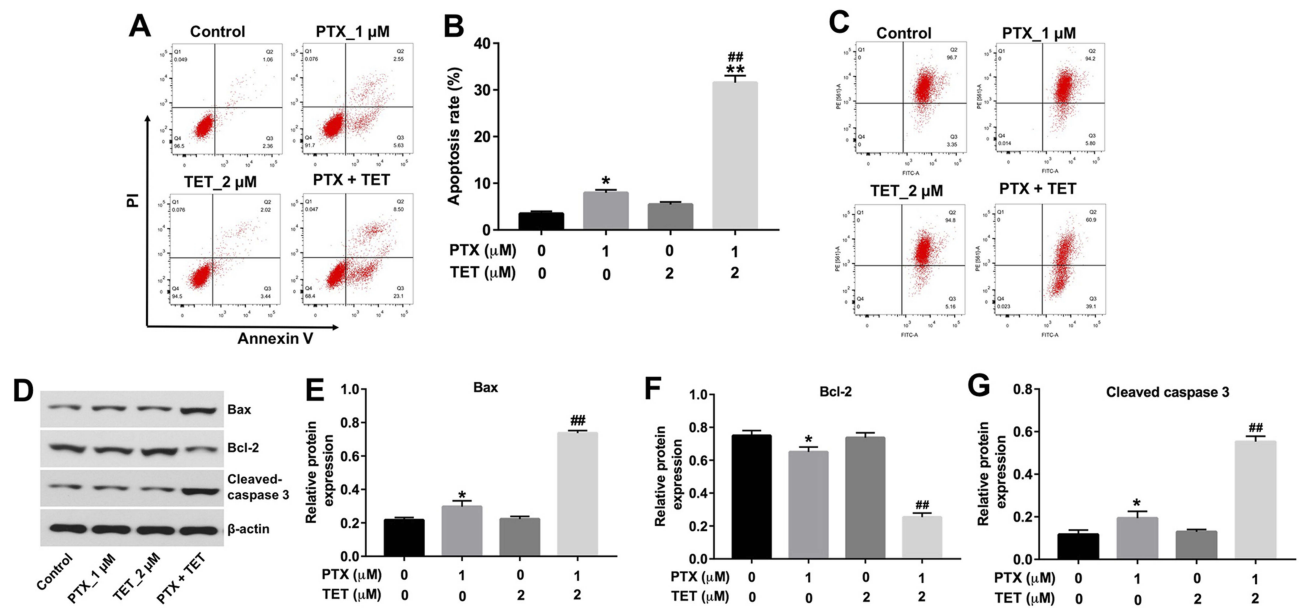
TET Enhanced the Sensitivity of SKOV3/PTX Cells to PTX by Inhibiting the β-Catenin/c-Myc/Cyclin D1 Signaling Pathway

The effect of TET and PTX on cell cycle progression was determined by flow cytometry and Western blotting. As shown in Figure 4A and B, the percentage of cells in the
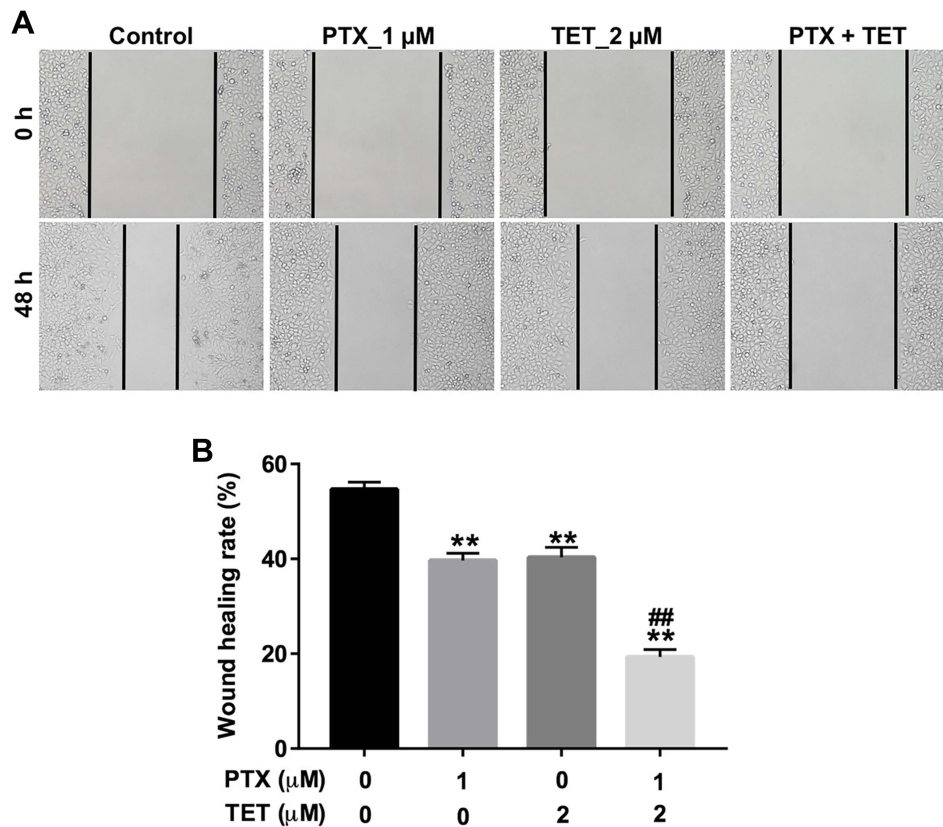
Figure 2 TET enhanced the pro-apoptotic effect of PTX in SKOV/PTX cells. SKOV3/PTX cells were treated with PTX (1 μM) or/and 2 μM TET for 72 h. (**A, B**) Apoptotic cells were detected by Annexin V/PI double staining and flow cytometry in SKOV3/PTX cells. (**C**) JC-1 staining was used to determine MMP loss. (**D**) Expression levels of Bax, Bcl-2 and cleaved caspase 3 in SKOV3/PTX cells were detected with Western blotting. (**E–G**) The relative expressions of Bax, Bcl-2 and cleaved caspase 3 were quantified via normalization to β-actin. *P<0.05, **P<0.01 compared with control group; ##P<0.01 compared with PTX_1 μM group.

Figure 3 TET enhanced the anti-migratory effect of PTX in SKOV3/PTX cells. SKOV3/PTX cells were treated with PTX (1 μM) or/and 2 μM TET for 72 h. (**A, B**) Cell migration was determined by wound healing assay. **P<0.01 compared with control group; ##P<0.01 compared with PTX_1 μM group.
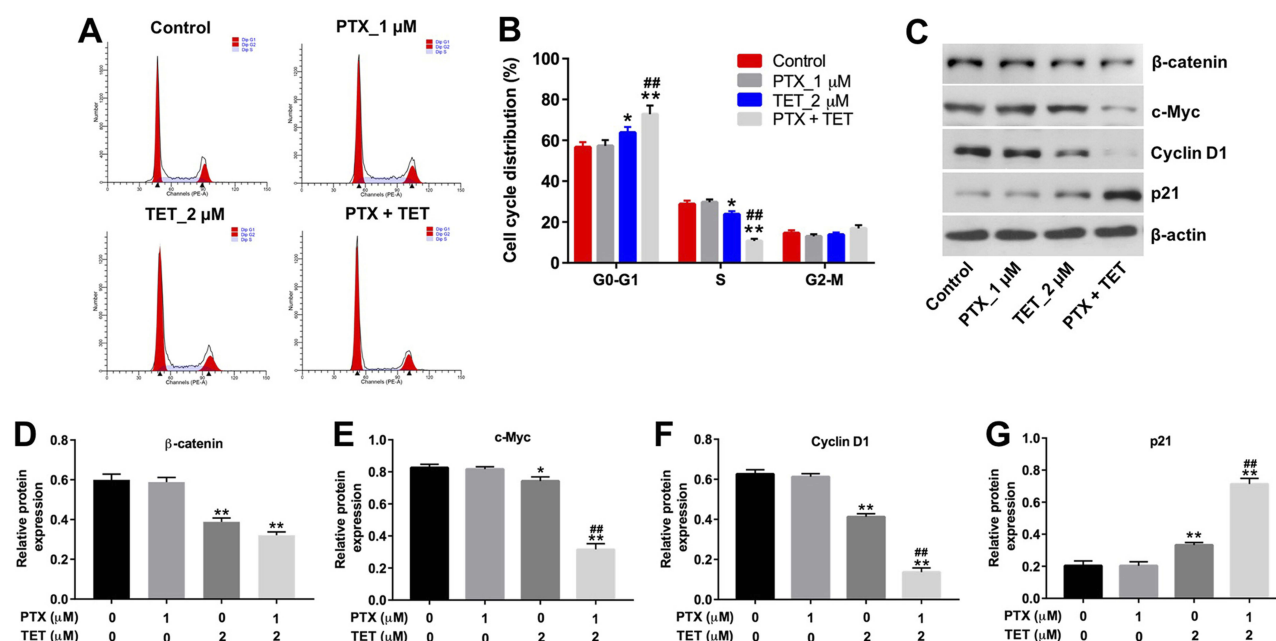
Figure 4 TET enhanced the sensitivity of SKOV3/PTX cells to PTX by inhibiting the β-catenin/c-Myc/Cyclin D1 signaling pathway. SKOV3/PTX cells were treated with PTX (1 μM) or/and 2 μM TET for 72 h. (**A**, **B**) Flow cytometry was used to measure cell cycle phase. (**C**) Expression levels of c-Myc, cyclin D1 and p21 in SKOV3/PTX cells were detected with Western blotting. (**D–G**) The relative expressions of β-catenin, c-Myc, cyclin D1 and p21 were quantified via normalization to β-actin. *P<0.05, **P<0.01 compared with control group; ##P<0.01 compared with PTX_1 μM group.

G0-G1 phase was obviously increased, with a concomitant decrease of cells in the S phase in the combination treatment group, compared to PTX treatment group. In addition, PTX had very limited effects on the expressions of β-catenin, c-Myc, cyclin D1 and p21 in SKOV3/PTX cells, whereas TET or combination treatment significantly downregulated the expressions of β-catenin, c-Myc and cyclin D1 and upregulated the level of p21 in SKOV3/PTX cells (Figure 4C–G). Furthermore, combination treatment significantly inhibited the proliferation of SKOV3/PTX cells via inducing apoptosis, which were markedly reversed in the presence of R-spondin 1 (β-catenin signal pathway activator) (Supplementary Figure 2A–C). These results indicated that TET could attenuate PTX resistance in SKOV3/PTX cells via inhibiting the β-catenin/c-Myc/Cyclin D1 signaling pathway.
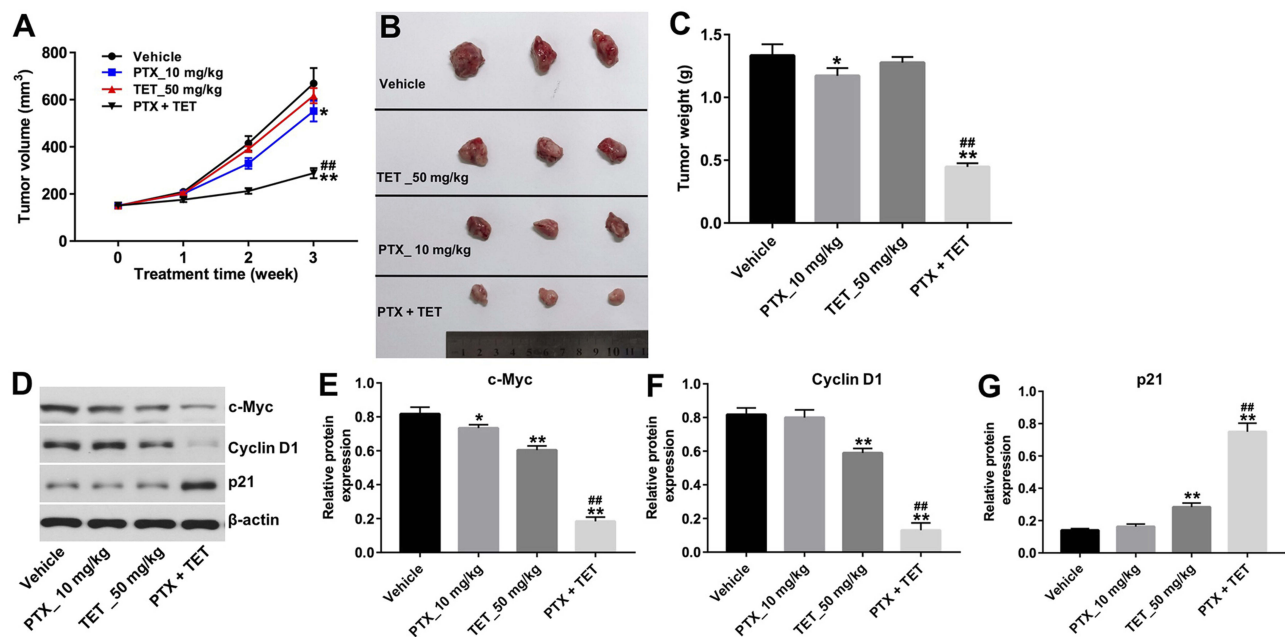
TET Enhanced Anti-Tumor Effect of PTX in SKOV3/PTX Xenograft

Cell line-derived xenograft model was established to investigate the effect of TET and PTX on ovarian tumor growth in vivo. As indicated in Figure 5A–C, the tumor volume and tumor weight in PTX group was significantly decreased compared with control group, which were further reduced in the presence of TET. In addition,

Western blotting results indicated that combination of PTX with TET treatment significantly downregulated the levels of c-Myc and Cyclin D1, but notably upregulated the expression of p21 in tumor tissues compared with PTX treatment group (Figure 5D–G). These results illustrated that TET could enhance the anti-tumor effect of PTX in SKOV3/PTX xenograft.

Discussion

Chemotherapy plays an important role in the treatment of human cancers, whereas drug resistance is one of the major challenges in the fight against ovarian cancer.[10,21] Recently, traditional Chinese medicine (TCM) combined with conventional chemotherapeutic agents has received growing attention.[22] Previous studies indicated that combination of TCM and chemotherapeutic drugs could overcome drug resistance.[23,24] Clinically, patients with advanced non-small cell lung cancer received TET combined with gemcitabine and cisplatin treatment. The results indicated that TET could mitigate adverse reactions to chemotherapy for patients with advanced non-small cell lung cancer.[25] However, the effect of the combination of PTX and TET on PTX-resistance ovarian cancer cells remains unclear. In this study, we found that combination of TET and PTX significantly enhanced the anti-tumor effect of PTX in vitro and in vivo.

Figure 5 TET enhanced anti-tumor effect of PTX in SKOV3/PTX xenograft. SKOV3/PTX cells were transplanted subcutaneously into nude mice to establish a xenograft model of ovarian cancer. (**A**) Subcutaneous tumor xenograft volumes were monitored and measured weekly. (**B**) Representative photographs of SKOV3/PTX xenograft tumor were captured on day 21. (**C**) Tumor weights in each group were calculated. (**D**) Expression levels of c-Myc, cyclin D1 and p21 in tumor tissues were detected with Western blotting. (**E–G**) The relative expressions of c-Myc, cyclin D1 and p21 were quantified via normalization to β-actin. *P<0.05, **P<0.01 compared with vehicle group; ##P<0.01 compared with PTX_10 mg/kg group.

Previous study indicated that TET exerted multiple biological activities on cancer cells, such as induction of apoptosis, inhibition of proliferation and reversal of multi-drug resistance.[26] Wang et al found that TET enhanced the sensitivity of drug-resistant esophageal squamous carcinoma cells to cisplatin via suppressing of multidrug resistance-associated protein 1.[27] Zou et al indicated that tetramethylpyrazine enhances the anti-tumor effect of PTX in ovarian cancer via inducing apoptosis.[10] In this study, TET enhanced the inhibitory effects of PTX on the proliferation and migration of SKOV3/PTX cells. In addition, TET enhanced the pro-apoptotic effect of PTX in SKOV3/PTX cells via downregulating the expression of Bcl-2, and upregulating the levels of Bax and active caspase 3, which were consistent with previous studies. Meanwhile, combination of PTX with TET notably inhibited the tumor growth in SKOV3/PTX xenograft. These data suggested that TET could enhance the anti-tumor effect of PTX in SKOV3/PTX cells in vitro and in vivo.

Previous study found that overexpression of c-Myc is intently associated with cell proliferation, while downregulation of c-Myc could inhibit the cell cycle progression.[28] In addition, evidence has been shown that p21 functioned as a negative regulator of cell cycle progression at G1 and S phase.[29] Meanwhile, cyclin D1 is an important regulator in the G1 phase of the cell cycle, increasing the level of Cyclin D1 might contribute to tumorigenesis.[30] Zhang et al found that combination of TET with PTX markedly induced apoptosis in ovarian cancer cells via inhibition cell cycle progression.[18] Meanwhile, Hou et al indicated that TET enhanced the anti-tumor effect of epirubicin on breast carcinoma multi-drug resistance cells.[31] Our results found that 1 μM PTX had very limited effect on cell cycle distribution in SKOV3/PTX cells. In addition, 2 μM TET markedly upregulated the level of p21 and downregulated the levels of c-Myc and Cyclin D1 in SKOV3/PTX cells. Singh et al indicated that TET inhibited the growth of pancreatic cancer cells via increasing the level of p21 and decreasing the level of Cyclin D1, which was consistent with our results.[32] Meanwhile, Sánchez-Carranza et al found that gallic acid reduced PTX resistance in ovarian carcinoma cells via inducing G2/M cell arrest.[33] In this study, by reducing the expressions of cyclin D1 and c-Myc and increasing the level of p21, combination of TET and PTX significantly increased the distribution of G1 phase in cell cycle in SKOV3/PTX cells, and thus induced G1 phase cell cycle arrest. These data indicated that TET enhanced the sensitivity of SKOV3/PTX cells to PTX via inhibiting cell cycle transition at G1/S boundary.

Evidence has been shown that β-catenin, an important transduction factor in the Wnt signaling pathway, plays a vital role in regulating chemoresistance in epithelial ovarian cancer.[34] In addition, β-catenin involved in the regulation of cell growth, cell cycle, and apoptosis.[35] Moreover, inhibition of β-catenin in cancer cells could suppress the cell cycle distribution via downregulating the expressions of Cyclin D1 and c-Myc.[35] Our data indicated that PTX had no effects on the expressions of β-catenin, c-Myc, and Cyclin D1 in SKOV3/PTX cells, whereas TET or combination treatment significantly downregulated the expressions of β-catenin, c-Myc and cyclin D1 in SKOV3/PTX cells, indicating that TET enhanced anti-tumor effect of PTX in SKOV3/PTX cells via inhibition the β-catenin/c-Myc/Cyclin D1 signaling pathway. Meanwhile, Liao et al found that TET could reverse multi-drug resistance in cancer cells via downregulating the expression of ABCB1.[36] In the future, the association between β-catenin and ABCB1 in SKOV3/PTX cells should be investigated, which may further confirm the roles and mechanisms of TET in regulating ovarian cancer cells resistance.

Conclusion

In this study, we found that TET could enhance the sensitivity of SKOV3/PTX cells to PTX via inducing apoptosis and cell cycle arrest. In addition, TET enhanced anti-tumor effect of PTX in SKOV3/PTX cells via inhibition of the β-catenin/C-myc/Cyclin D1 signaling pathway. Therefore, PTX combined with TET might be considered as a potential approach for the treatment of PTX-resistant ovarian cancer.

Disclosure

The authors declare no competing financial interests.

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