Triptolide exhibits antitumor effects by reversing hypermethylation of WIF-1 in lung cancer cells

XIAOLIANG MAO, JICHUN TONG, YONG WANG, ZHENG ZHU, YAJUN YIN and YEMING WANG

Department of Cardiothoracic Surgery, Changzhou No. 2 People's Hospital,
The Affiliated Hospital of Nanjing Medical University, Changzhou, Jiangsu 213000, P.R. China

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Abstract. Triptolide (TP) exhibits numerous biological activities, including immunosuppressive, anti-inflammatory and antitumor effects. The aim of the present study was to investigate the role of TP as a potent therapeutic drug for the treatment of lung cancer and to investigate the underlying therapeutic mechanisms. Western blot analyses and reverse transcription-quantitative polymerase chain reaction (PCR) were performed to investigate the expression of genes at transcriptional and translational levels, respectively. Methylation-specific PCR assays were conducted to investigate whether TP affects the Wnt inhibitory factor-1 (WIF-1) methylation status and subsequently affects apoptosis, migration or the invasion of lung cancer cells. The results of the present study revealed that the methylation status of WIF-1 in lung cancer cell lines A549 and H460 was significantly enhanced compared with the human normal bronchial epithelial cell line HBE, whereas treatment with TP was revealed to induce the demethylation of WIF-1. The present study aimed to investigate whether the biological activities of TP are regulated by inhibiting the Wnt signaling pathway via an increase in WIF-1 expression levels. The results of the present study revealed that Wnt signaling was suppressed in cells following treatment with TP, which was concluded by the downregulation of Axin 2 and β-catenin expression. Further investigation demonstrated that the silencing of WIF-1 expression with small interfering RNA reversed the TP-induced upregulation of WIF-1 expression, upregulated Axin 2 and β-catenin expression and enhanced the activation of Wnt signaling. Notably, an upregulation of cellular tumor antigen p53 expression, and downregulation of matrix metalloproteinase-9 (MMP-9) and phosphorylated-nuclear factor-κB (NF-κB) P65 (p-P65) levels was observed following TP treatment. These results suggest that the Wnt, p53 and NF-κB signaling pathways mediate the potent antitumor effects of TP. Notably, the silencing of WIF-1 did not completely recover the levels of p53, MMP-9 and p-P65 in cells treated with TP compared with the control cells, thus suggesting that TP exhibits further functions in addition to the targeting of WIF-1.

Correspondence to: Dr Jichun Tong, Department of Cardiothoracic Surgery, Changzhou No. 2 People's Hospital, The Affiliated Hospital of Nanjing Medical University, 68 Middle Ge Lake Road, Changzhou, Jiangsu 213000, P.R. China
E-mail: erxz9657@126.com

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Introduction

Triptolide (TP) is a traditional Chinese medicine, apredominant active ingredient in *Tripterygium wilfordii* and is a diterpenoid compound (1). Numerous studies have suggested that TP can inhibit proliferation of numerous tumor cells, such as breast cancer, gastrointestinal cancer, prostate cancer and nervous system tumors (1-3). TP was discovered in 1972 and the molecular mechanism of its biological activities has been extensively studied (4). TP exhibits an immunomodulatory role, predominantly via inhibition of nuclear factor-κB (NF-κB), and subsequent suppression of the production of inflammatory factors and thus the immune response of the body (5,6). In addition, TP can induce apoptosis in numerous tumor cell types, predominantly via inhibition of heat shock transcription factor 1, activator protein 1 (AP-1), NF-κB and other transcriptional regulators, thus exhibiting an antitumor effect (5,7). Such results suggest that NF-κB may be associated with the underlying therapeutic mechanism of TP. Furthermore, these studies demonstrated that functional cellular tumor antigen p53 (p53) is necessary for the performance of the various biological activities of TP (6).

Lung cancer is the most common type of cancerous tumor globally. Despite the possibility of early diagnosis, chemotherapy, radiotherapy and immunotherapy, the 5-year survival rate of lung cancer remains <15% (8). Small molecule inhibitors, predominantly epidermal growth factor receptor-associated drugs, including gefitinib and erlotinib, have been reported to have significant antitumorigenic effects with fewer side effects (9,10). Despite the wide use of these drugs, there are significant drug resistances in patients with epidermal growth factor receptor-negative cancer (9,11). Thus, there is an urgent requirement to identify novel antitumor agents that can act on irregularly functioning signaling pathways. It has been reported that Wnt family proteins can regulate cell development and growth via regulation of downstream proteins (12). The

most predominant proteins associated with the Wnt signaling pathway, from the extracellular to intracellular environment, include the Wnt protein (predominantly encoding secretory glycoprotein), cell membrane Wnt receptor protein, β-catenin and the anaphase promoting complex protein (glycogen synthase-3β kinase, Axin and conductin) (13). Wnt signaling pathways are generally expressed during embryonic development, and Wnt signaling is subsequently downregulated or absent in mature organisms (14). However, numerous studies have revealed that abnormal expression of Wnt signaling activates the cell cycle, induces proliferation associated with proto-oncogenes and promotes tumorigenesis (15,16). Studies have also demonstrated that the expression of Wnt ligands are upregulated in lung cancer cell lines and tissues (17,18). Overexpression of Wnt ligands results in the ectopic activation of β-catenin in the cytoplasm/nucleus of lung cells and the activation of transcription of downstream target genes (predominantly encoding a number of proto-oncogenes and cell cycle regulators) (19). Despite the cause of Wnt ligand overexpression in lung cancer cells remaining unclear, one of the notable associated mechanisms is the absence of the Wnt antagonist protein (20). Wnt inhibitory factor-1 (WIF-1) is present in normal cells via binding to Wnt ligands to prevent over activation of the Wnt pathway (21). Studies have revealed that WIF-1 is suppressed in lung cancer cells, thus weakening its binding to Wnt ligands and inhibiting the sustained activation of the Wnt pathway (22,23). In addition, it has been reported that hypermethylation of the WIF-1 promoter is frequently present in lung cancer specimens, with suppressed or absent expression of WIF-1 (23). Therefore, one of the notable causes of abnormal activation of the Wnt pathway in lung cancer cells may be due to hypermethylation of the WIF-1 promoter region, thus resulting in the suppressed regulation of the Wnt ligand expression (22-24). Reversing the hypermethylation of the WIF-1 promoter in lung cancer cells and restoring the expression level of WIF-1 in lung cancer cells, and thus further inhibiting the activation of the Wnt pathway, are important therapeutic targets for the treatment of lung cancer requiring further investigation.

The aim of the present study was to investigate the effect of TP on the proliferation, invasion, migration and apoptosis of human lung cancer cells, and to determine the underlying molecular mechanism of the therapeutic effects of TP on lung cancer cells. The results of the present study will aim to provide a theoretical basis for further study regarding the anticancer effect of TP.

Materials and methods

Cell culture and treatment. The lung cancer cell lines A549, H460 and NCI-H446, and human normal bronchial epithelial cell line HBE, all purchased from the American Type Culture Collection (Manassas, VA, USA) were used in the present study. The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with a humidified atmosphere of 5% CO_2. The cells were cultured to 70-80% confluence prior to further investigation.

Methylation-specific polymerase chain reaction (PCR). A549, H460, NCI-H446 and HBE cells were harvested to detect WIF-1 methylation PCR. Following this, A549 and H460 cells were collected and incubated with 0.03, 0.3 and 3 μM TP for 24 h. The methylation-specific PCR protocol was performed as described previously (25). Briefly, the extracted DNA was treated with sodium bisulfite. This treatment ensured that unmethylated cytosine in the CpG nucleotides was converted to uracil, whereas the methylated cytosine remained unaffected. The modified reaction was performed using an EZ DNA Methylation-Gold kit (Zymo Research Corp., Irvine, CA, USA). Subsequently, the treated DNA was amplified using methylation-specific and non-methylation-specific primers. The thermocycling conditions for PCR amplification: Pre-denaturations (94˚C, 5 min); 35 cycles of denaturation (94˚C, 30 sec), annealing (56˚C, 30 sec) and extension (72˚C, 30 sec); post-extension (72˚C, 5 min). 2X PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, China) was used in these assays. WIF-1 methylation PCR primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The following methylation-specific primers were used: forward, 5'-GGGCGTTTTATTGGGCGTAT-3' and reverse, 5'-AAACCAACAATCAACGAAC-3'. The following non-methylation-specific primers were used: forward, 5'-GGG TGTTTTATTGGGTGTAT-3' and reverse, 5'-AAACCAACA ATCAACAAAAC-3'.

Cell proliferation assay. Cell proliferation was investigated via a Cell Counting kit-8 (CCK-8) assay. A549 and H460 cells ($5x10^4$ cells/well) were seeded in 96-well plates and incubated with 0.03, 0.3 and 3 μM TP for 6, 12 and 24 h time intervals. Subsequently, 20 μl of CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into the cells collected from 6, 12 and 24 h time intervals. Following incubation for 4 h, the absorptions of cells were determined at 450 nm using an ELISA reader (ELx800TM; BioTek Instruments, Inc., Winooski, VT, USA).

Transwell cell migration assay. A549 cells were digested with 0.25% Trypsin-EDTA solution, harvested and washed twice to remove serum. Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich; Merck KGaA) and then adjusted to $2x10^5$/ml. Subsequently, 300 μl cell solution was added to upper chambers (BD Biosciences, Franklin Lakes, NJ, USA) and then placed on 24-well plate for 2 h. Lower chambers were filled with 500 μl DMEM medium containing 2.5% FBS and incubated for 24 h. Following incubation, migratory cells were fixed with 4% formaldehyde for 15 min at room temperature and then stained with 0.1% crystal violet dyes for 3 h at room temperature. A total of 5 fields of view were selected randomly, observed and imaged under a light microscope (magnification, x400; 80i; Nikon Corporation, Tokyo, Japan). Absorbance values at 570 nm were measured using SpectraMax® M5/M5e (Molecular Devices, LLC, Sunnyvale, CA, USA). The migration rate was expressed as the percentage of the control.

Transwell cell invasion assay. Cell migration analysis was performed using an ECM554 invasion kit

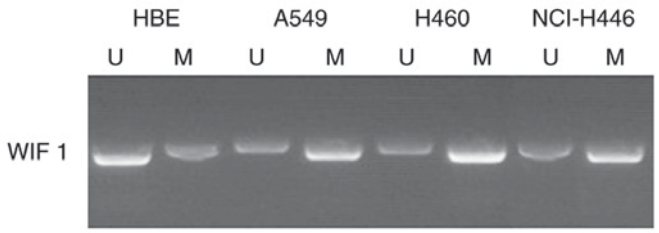

Figure 1. The hypermethylation of WIF-1 promoter in lung cancer cells is enhanced compared with the normal bronchial epithelial cell line HBE cell line. Methylation-specific polymerase chain reaction assay was performed. The results demonstrated that the methylation status in cancer cells was enhanced compared withthe normal bronchial epithelial cell line HBE. U, unmethylated; M, methylated; WIF 1, Wnt inhibitory factor-1.

(Chemicon International; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, cells from all experimental groups were collected and resuspended in DMEM medium and then adjusted to $2x10^5$ /ml. The upper chambers of the Transwell was placed on 24-well plate and 300 μl serum free medium was added, and the bottom chambers were filled with DMEM medium containing 2.5% FBS. Following incubation for 10 min, 250 μl cell solution was used to replaced the DMEM in the upper chambers and then underwent further incubation for 24 h. Invaded cells were stained using 0.1% crystal violet dye for 3 h at room temperature. A total of 5 fields of view were selected randomly, observed and imaged under microscope (magnification, x400; 80i; Nikon Corporation). The invasion rate was expressed as the percentage of the control.

Cell transfection. At 80% confluence A549 cells were transfected with WIF1 small interfering (si)RNA sense, 5'-CCUGUCAAU AUCCAUUCCAUU-3' and antisense, 5'-UGGAAUGGAUAU UGACAGGUU-3'; negative siRNA control sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3') via Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The medium was replaced with complete DMEM containing 10% FBS (Sigma-Aldrich; Merck KGaA) following incubation for 5 h at 37˚C.

Western blot assay. A549 cells were collected and then lysed in radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.25% deoxycholate, protease and phosphatase inhibitors. Total protein concentration was determined using the bicinchoninic acid assay (Hyclone; GE Healthcare, Chicago, IL, USA). Total protein (20-40 μg) was separated via electrophoresis using 10% SDS-PAGE gel and the proteins were then transferred electrophoretically onto polyvinylidene fluoride membranes. Following blocking with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with primary antibodies WIF1 (1:1,000; ab224335), p53 (1:1,000; ab26), MMP-9 (1:1,000; ab38898), Axin2 (1:1,000; ab109307), p-P65 (1:1,000; ab176647), P65 (1:1,000; ab16502), β-catenin (1:5,000; ab32572) and β-actin (1:1,000; ab8226; all Abcam, Cambridge, MA, USA) overnight at 4˚C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilutions; NA934V; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Following washing with TBST, the blots were developed using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences) for 2 h at room temperature. β-actin was used as an internal control. Band intensities were quantified by densitometry using ImageJ software (ver 1.45; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative PCR assay (RT-qPCR). A549 cells were collected and total RNA was isolated using the RNeasy mini-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The RNA was then reverse transcribed to cDNA using NCode VILO miRNA cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific). The temperature conditions for RT were as follows: 30˚C for 10 min, 42˚C for 30 min, 99˚C for 5 min, 4˚C for 5 min. qPCR was performed using iQ SYBR-Green Supermix on the iCycleriQ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions for qPCR: 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All assays were performed in duplicate. Gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (26). Relative mRNA expression was normalized to β-actin expression. Primers used in this study were as follows: WIF-1 forward, 5'-ATGAATTCCTGTCCTTGC GC-3' and reverse, 5'-TCCACTTCAAATGCTGCCAC-3'; β-actin forward, 5'-CCCTGGAGAAGAGCTACGAG-3' and reverse, 5'-CGTACAGGTCTTTGCGGATG-3'.

Statistical analysis. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis. All data are expressed as mean ± standard deviation. Results were analyzed by two-tailed and unpaired Student's t-tests. One-way analysis of variance was used for comparison of multiple (>2) groups and Tukey's post hoc test for pairwise comparisons was used in this study. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypermethylation of the WIF-1 promoter is enhanced in lung cancer cell lines. To investigate whether TP has the ability to reverse the hypermethylation of the WIF-1 promoter, the methylation status of the WIF-1 promoter in non-small cell lung cancer A549, H460 and NCI-H446 cell lines was determined. The results revealed that the WIF-1 promoter was hypermethylated in A549, H460 and NCI-H446 cells, and that there was suppressed methylation in HBE cells compared with lung cancer cell lines (Fig. 1). Due to limitations in funding, only A549 and H460 cells were selected for subsequent analysis.

TP has a demethylation effect on the WIF-1 promoter in A549 and H460 cells. In order to investigate whether treatment with TP has a demethylation effect on the WIF-1 promoter in A549 and H460 cells, an *in vitro* intervention experiment was performed. The results revealed that treatment with TP had a demethylation effect on A549 cells (Fig. 2A) and H460 cells (Fig. 2B) in a dose dependent manner 24 h post-treatment compared with the control.

TP suppresses the proliferation of A549 cells. Previous studies have revealed that TP has potent tumor cytotoxicity (1-3). Therefore, the viability of A549 cells following treatment with TP was investigated. The results presented in Fig. 3A demonstrated that treatment with TP significantly suppressed the viability of A549 cells in a dose- and time-dependent manner, and that the relative cell viability reached 31.07% 24 h post-treatment with 3 μM TP compared with the control.

TP significantly inhibits the invasion and migration of A549 cells. Compared with the control, the invasion and migration of A549 cells were markedly suppressed 24 h post-treatment with 0.03, 0.3 and 3 μM TP (Fig. 3B and C). As revealed in Fig. 3B, the migration of A549 cells was significantly attenuated following treatment with TP in a dosage-dependent manner. Similarly, the invasion of A549 cells was significantly suppressed following treatment with TP (0.3 and 0.03 μM) compared with the control (Fig. 3C).

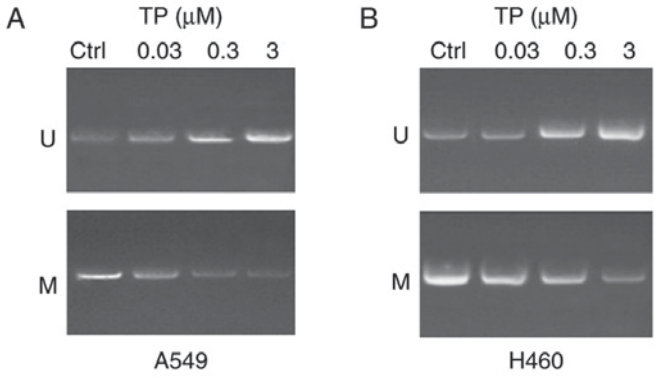

Figure 2. Treatment with TP exhibits a marked demethlation effect on the WIF-1 promoter. Methylation-specific polymerase chain reaction assays demonstrated that the demethylation of the WIF-1 promoter was enhanced in a dose-dependent manner in (A) A549 and (B) H460 cells. U, unmethylated; M, methylated; WIF 1, Wnt inhibitory factor-1; TP, triptolide; Ctrl, control.

Demethylation of the WIF-1 promoter following treatment with TP increases the expression of WIF-1. To investigate the effects of TP on the expression levels of WIF-1in A549 cells, the mRNA and protein levels of WIF-1 were investigated. At 24 h post-treatment, the expression of WIF-1 protein in A549 cells was enhanced in a dose-dependent manner (Fig. 4A). The expression level of WIF-1 in cells treated with 3 μM TP was ~20-fold that of the control. Furthermore, the WIF-1 mRNA expression level in A549 cells was significantly increased following TP treatment in a dose-dependent manner (Fig. 4B).

TP affects the expression of genes associated with apoptosis and proliferation. The results of the present study revealed that TP is associated with the expression of WIF-1 and the viability of A549 cells. Therefore, the present study aimed to investigate whether increased levels of WIF-1 expression, enhanced by treatment with TP, could inhibit the Wnt signaling pathway. Furthermore, the present study aimed to investigate whether treatment with TP affects the expression levels of matrix metalloproteinase-9 (MMP-9) and p53, as well as the activation of NF-κB P65, which are associated with cell migration, apoptosis and survival. A notable increase regarding p53 expression was observed following treatment with TP (3 μM) compared with control (Fig. 4C and D). By contrast, the expression level of MMP-9 was significantly decreased in cells treated with TP (Fig. 4C and D). The expression levels of β-catenin and Axin2, two key molecules of the Wnt pathway, were significantly decreased following treatment with TP compared with the control (Fig. 4C and D). Notably, the level of phosphorylated-P65 was also significantly decreased following treatment with TP (Fig. 4C and D). These results suggest that the Wnt and NF-κB signaling pathways were strongly suppressed following treatment with TP.

To further investigate the role of WIF-1 in the regulatory effect of TP on Wnt and NF-κB pathways, WIF-1 expression was silenced by siRNA. As demonstrated in Fig. 5A and B, TP-induced elevations in levels of WIF-1 protein and mRNA were clearly downregulated compared with the control and siRNA negative control. The expression of p53 was significantly decreased in cells treated with TP+siRNA compared with cells treated with TP alone or TP+NC (Fig. 5C). However, the level of p53 in cells treated with TP+siRNA was enhanced compared with the control. By contrast, MMP-9 expression in TP+siRNA cells was markedly enhanced compared with cells treated with TP alone or TP+NC, and lower than that exhibited by the control (Fig. 5C). In cells treated with TP+siRNA, the expression levels of Axin 2 and β-catenin were recovered to a similar level exhibited by the control, and significantly elevated compared with cells treated with either TP alone or TP+NC (Fig. 5D). There were no significant differences in levels of p-P65 exhibited among cells treated with TP+siRNA, TP alone or TP+NC (Fig. 5D). These results suggest that TP not only suppresses the Wnt pathway by increasing WIF-1 expression, but also inhibits the NF-κB pathway (which is not regulated by WIF-1).

Discussion

TP, a traditional Chinese medicine isolated from *Tripterygium wilfordii*, has been used to treat inflammation and autoimmune diseases (27). Numerous studies have demonstrated that TP exhibits antitumor effects on various cancer cell lines, including bladder, liver, cervical and breast cancer cell lines (28,29). To investigate the effect of TP on the viability, invasion and migration of lung cancer cells, human lung cancer cell lines A549 and H460 were treated with TP. The results revealed that treatment with TP markedly suppressed the viability, invasion and migration of lung cancer cells. Furthermore, the results demonstrated that treatment with TP increased the expression levels of WIF-1 via demethylation of the WIF-1 promoter. Further investigation revealed that TP also increased the expression of p53, and decreased the levels of MMP-9 and p-P65, which are associated with cell apoptosis, proliferation, survival, invasion and migration (1-4).

The Wnt signaling pathway has an important function in the development of the embryo and is associated with the regulation of cell proliferation, differentiation and migration (30,31). Hypermethylation of the WIF-1 promoter is associated with excessive activation of the Wnt signaling pathway in human lung cancer (24,32-34). Thus, restoring

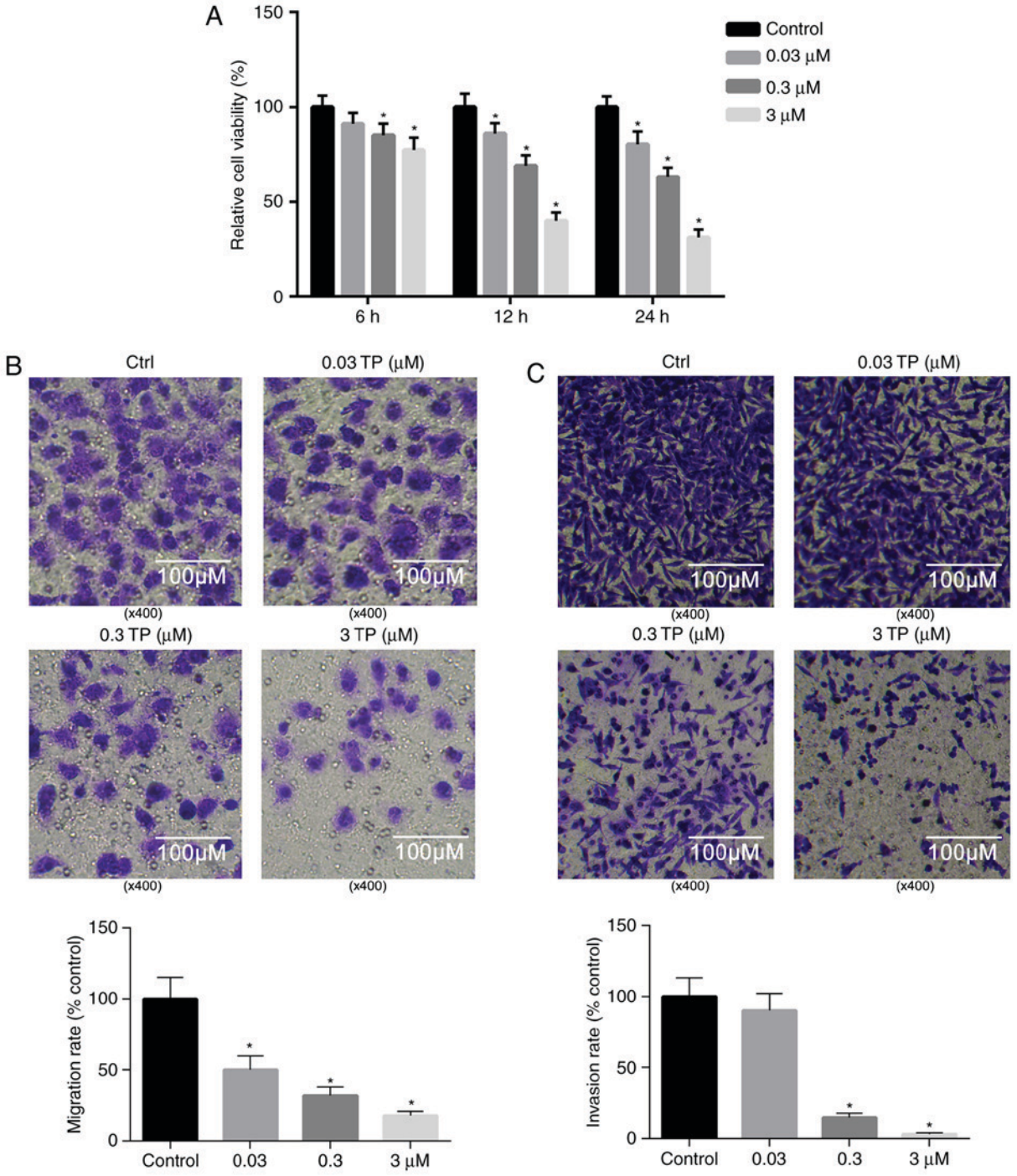

Figure 3. Treatment with TP affects cell viability, migration and invasion in A549 cells. (A) Cell Counting kit-8 assays demonstrated that TP suppressed the cell viability in a dosage- and time-dependent manner. (B) Transwell cell migration assay revealed that cell migration was significantly inhibited 24 h post-treatment of cells with TP (0.03, 0.3 and 3 μM). (C) Transwell cell invasion assay demonstrated that cell invasion of A549 cells was significantly suppressed following treatment with TP, particularly when TP concentration was 3 μM. TP, triptolide; Ctrl, control. *P<0.05 vs. control.

normal WIF-1 expression may downregulate the Wnt signaling pathway and inhibit the progression of lung cancer. In human tumors, abnormal methylation of CpG islands in promoter regions results in a suppression of the transcriptional activity of tumor suppressor genes (35-37). Previous studies have demonstrated that numerous tumor suppressor genes associated with lung cancer are downregulated via methylation of the promoter region (23,35,38). Previous studies had also revealed that WIF-1 is silenced by hypermethylation of the associated promoter in lung cancer cell lines and lung cancer surgical specimens (22,38). In addition, administration of the classic demethylation drug 5-aza-2'-deoxycytidine (DAC) demethylates the WIF-1 promoter to restore WIF-1 expression and thus restores downregulation of the activity of the Wnt signaling pathway (39,40). Despite DAC exhibiting a well-established demethylation effect, its therapeutic use is limited by its toxic effects on cells (41,42). TP is a natural compound that exhibits low cytotoxicity and a potential demethylation function, and thus may represent a novel therapeutic agent for the treatment of patients with

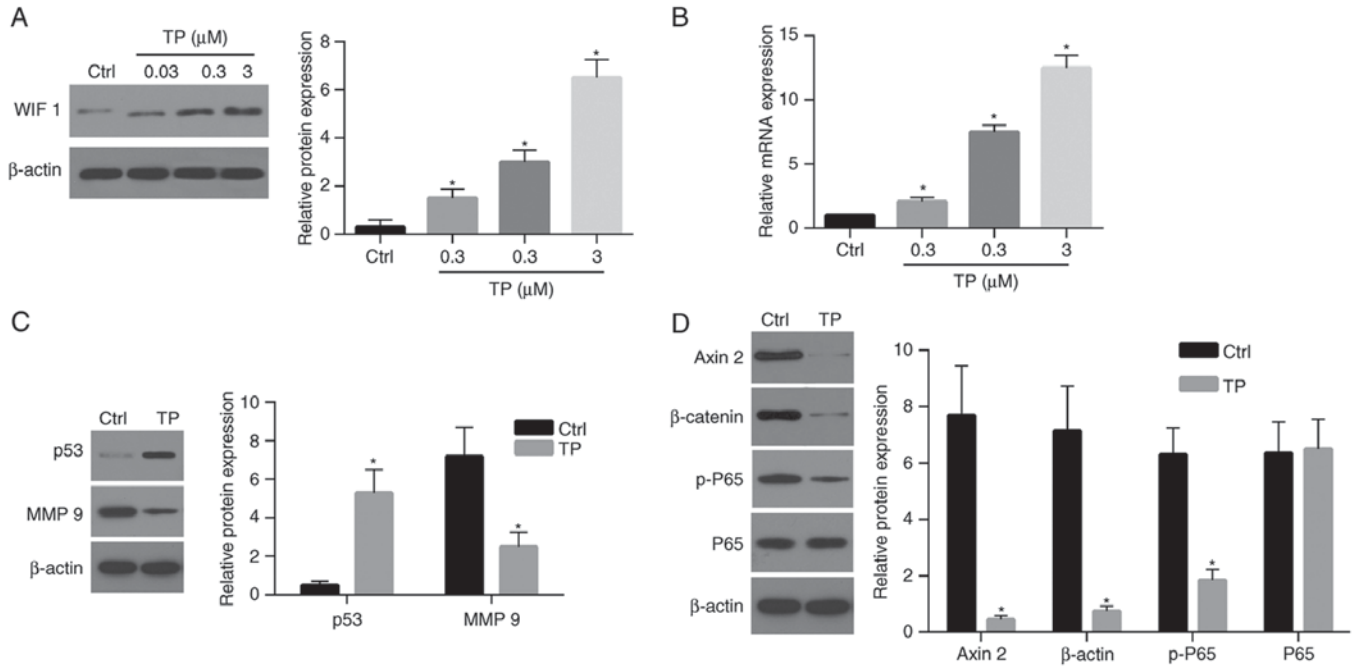

Figure 4. TP affects the expression of numerous genes associated with apoptosis, cell proliferation, migration and invasion. (A) Protein and (B) mRNA expression levels of WIF-1 were upregulated following treatment with TP in a dosage-dependent manner. Western blot assays demonstrated that the expression level of (C) p53 was significantly increased in cells treated with TP (3 μM) compared with the control, and the level of MMP 9 was significantly decreased compared with the control. (D) The levels of Axin 2, β-catenin and p-P65 in cells treated with TP (3 μM) were significantly decreased compared with the control. TP, triptolide; Ctrl, control; WIF 1, Wnt inhibitory factor-1; MMP-9, matrix metalloproteinase-9; p-, phospho-; P65, NK-κB P65. *P<0.05 vs. control.

lung cancer. The results of the present study revealed that treatment with TP significantly suppressed the viability, invasion and migration of A549 and H460 cells. In addition, the results demonstrated that TP markedly suppressed the degree of methylation of the WIF-1 promoter, thus inducing upregulation of WIF-1 expression. It was hypothesized that TP suppresses cell proliferation, invasion, migration and apoptosis by inhibiting the Wnt signaling pathway via upregulation of WIF-1 expression. Downregulated expression of Axin 2 and β-catenin suggested that treatment with TP suppresses the Wnt signaling pathway. Furthermore, an increase in p53 expression and a reduction in MMP-9 expression were observed, the results of which were consistent with the inhibition effect of TP on cell proliferation, migration and apoptosis.

TP had been demonstrated to exhibit significant growth inhibitory effects on various solid tumors such as breast cancer, gastrointestinal cancer, prostate cancer and nervous system tumors (43-45). TP has entered Phase I clinical trials, for the treatment of leukemia (46,47). It has been revealed that TP exhibits an antitumor effect predominantly via inhibition of numerous transcriptional regulators such as NF-κB and AP-1, in which the NF-κB pathway is one of the most important antitumor targets (5,29). P65 is an important heterodimer of NF-κB. Inhibitor of κB (I-κB) and NF-κB exist in the form of inactive complexes in the cytoplasm. Following activation, the NF-κB/I-κB complex dissociates and releases free NF-κB into the nucleus to regulate the transcription of the target genes (48-50). Notably, the level of p-P65 was downregulated in cells post-treatment with TP, which is consistent with the observations of previous studies (51-53). Recently, similar studies determined that TP not only inhibits NF-kB nuclear translocation in human lung adenocarcinoma paclitaxel-resistant cell line A549/Taxol, but also inhibits the function of RNA polymerase II (54,55).

The results of the present study revealed that knockdown of WIF-1 with siRNA resulted in a significant reduction of WIF-1 expression. As hypothesized, compared with cells treated with TP, the expression levels of Axin 2 and β-catenin in cells with additional siRNA treatment were increased and recovered to that exhibited by the control (cells without treatment), thus suggesting that the Wnt signaling pathway was activated. Silencing WIF-1 blocked TP-induced inhibition of the Wnt signaling pathway, thus suggesting that TP-induced inhibition of Wnt signaling pathway is WIF-1 dependent. Despite activation of Wnt signaling simulated by knockdown of WIF-1 not completely recovering the expression levels of p53, MMP-9 and p-P65 in cells treated with TP compared with the control, the results do suggest that treatment with TP affects p53 and NF-κB signaling pathways. Consistent with the results of the present study, Jiang *et al* (6) demonstrated that TP has anti-inflammatory and antitumor functions via suppression of cell proliferation, induction of apoptosis and downregulation of NF-κB and AP-1 transcriptional activity. In addition, previous studies have demonstrated that p53 activity is important for the anti-inflammatory, antitumor and pro-apoptotic effects of TP treatment on human gastric cancer cells (6,56).

The present study was limited due to funding; only A549 and H460 cells were investigated. Therefore, the findings of the present stud require further investigation with additional cell lines and analysis *in vivo*. In conclusion, the results of the present study suggested that promoter hypermethylation of WIF-1 is responsible for the abnormal

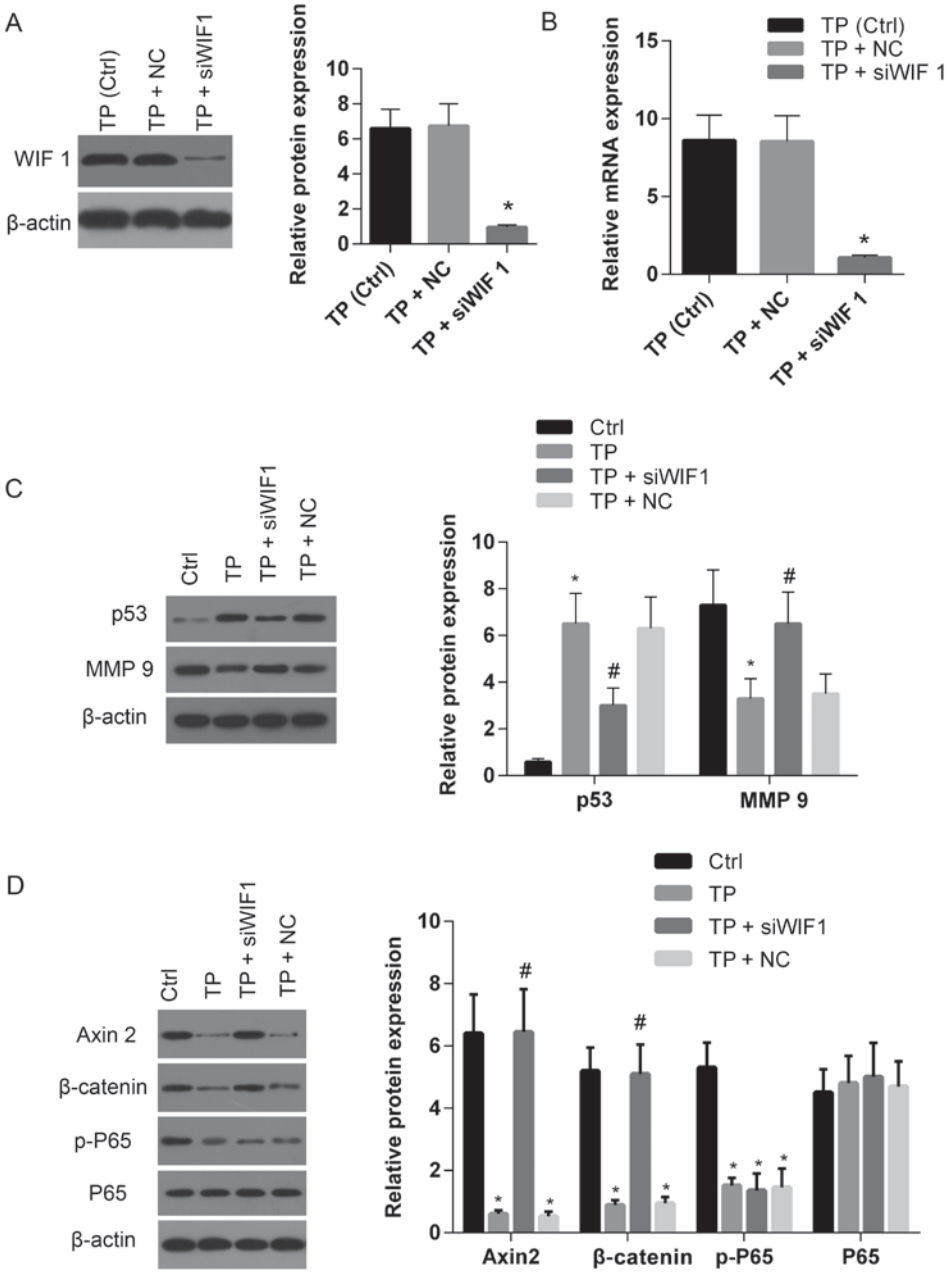

Figure 5. Silencing WIF-1 expression partially attenuates the effect of TP on the expression of several genes associated with apoptosis and cell proliferation, migration and invasion. The (A) protein and (B) mRNA expression levels of WIF-1 in cells treated with TP (3 μM) were downregulated following treatment with siWIF 1 compared with cells treated with TP alone or the negative control. (C) The protein levels of p53 and MMP-9 in cells treated with TP+siWIF 1 were markedly enhanced and suppressed compared with the control, respectively; whereas the protein levels of p53 and MMP-9 in cells treated with TP+siWIF 1 were markedly suppressed and enhanced compared with cells treated with TP alone. (D) The protein levels of Axin 2 and β-catenin in cells treated with TP+siWIF 1 were restored to the associated levels exhibited by the control, whereas there was no significant change regarding the level of p-P65 in cells treated with TP+siWIF 1 compared with cells treated with TP alone or negative control (TP+NC). TP, triptolide; Ctrl, control; NC, negative control; si, small interfering RNA; WIF 1, Wnt inhibitory factor-1; MMP 9, matrix metalloproteinase 9; p-, phospho-; P65, NK-κB P65. *P<0.05 vs. control. #P<0.05 TP+siWIF 1 vs. TP+NC or TP.

activation of Wnt signaling in lung cancer cells. TP exhibits significant inhibition on Wnt signaling via demethylation of the WIF-1 promoter. Silencing WIF-1 with siRNA reversed TP-induced inhibition of Wnt signaling, thus suggesting that TP-induced suppression of Wnt signaling is WIF-1 dependent. Furthermore, upregulation of p53, and downregulation of MMP-9 and p-P65, was observed in cells treated with TP. Notably, silencing WIF-1 did not restore the levels of p53, MMP-9 and p-P65 in cells treated with TP to those exhibited by the control, thus suggesting that p53 and NF-κB signaling pathways also have important roles in the antitumor effect of TP on lung cancer cells.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

XM and JT wrote the manuscript. XM conducted cell culture and treatments, cell proliferation and Transwell cell migration assays, and reverse transcription-quantitative polymerase chain reaction and western blot analyses. YoW and ZZ performed cell culture and treatment, cell proliferation assay, cell transfection and Transwell cell migration assay. YY and YeW performed methylation-specific polymerase chain reaction and reverse transcription-quantitative polymerase chain reaction. XM and JT made substantial contributions to the design of the study. XM and YoW analysed data. XM and JT critically revised the manuscript for important intellectual content. All authors read and approved the final the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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