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Original Article

Effect of Smac and Taxol on non-small-cell lung cancer

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A series of structurally unique second mitochondria-derived activator of caspases (Smacs) that act as antagonists of the inhibitor of apoptosis proteins (IAPs) directly have been discovered. They play crucial roles in mitochondrial apoptosis pathways and promote chemotherapy-induced apoptosis. In this study, we constructed a eukaryotic expression vector pcDNA3.1/Smac and transfected it into A549 human lung cancer cells. Then we analyzed the cell invasive and cloning ability, as well as cell apoptosis induced by Taxol. The results showed that over-expressed Smac significantly inhibited A549 cell invasive and cloning ability and promoted apoptosis following Taxol treatment. This finding provides a potential approach for the biological therapy of lung cancer.

Keywords second mitochondria-derived activator of caspase; non-small-cell lung cancer; invasive ability; apoptosis; cloning ability; chemosensitivity

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Introduction

The incidence of lung cancer is high, and the fatality rate is even higher due to the general ineffectiveness of conventional surgery and chemotherapy [1,2]. Approximately 80% of lung cancer patients are inoperable at diagnosis, and normal chemotherapy is unable to effectively prevent the growth of the tumor [3]. Resistance to apoptosis certainly plays a very relevant role in tumor development and progression. The ability of lung cancer cells to evade apoptosis is related to various biochemical properties, especially to up-regulation of antiapoptotic genes such as members of the inhibitor of apoptosis protein (IAP) family of proteins [4]. Ways to induce tumor apoptosis and improve the response to chemotherapy without affecting normal cells would be a major step forward. More recently, researchers discovered that second mitochondria-derived activator of caspase (Smac), an important gene in the mitochondrial apoptosis pathway, displays differential expression between normal and cancer tissues [5], with expression lost in tumor [6], suggesting that *Smac* is an anti-cancer or tumor suppressor gene. *Smac* mRNA expression has been reported to be significantly lower in lung cancer tissue than in normal lung tissue. Non-small-cell lung cancer (NSCLC) patients with higher *Smac* mRNA expression had significantly longer progression-free survival and overall survival with adjuvant chemotherapy [7–9]. Taxol is a novel anti-cancer drug. However, it generates low success rates, and like many conventional, non-targeted chemotherapeutics, it produces severe side effects. Therefore, new treatment regimens are required to increase the efficacy of Taxol. In this study, we investigated the role of Smac in Taxol-induced apoptosis of lung cancer cells *in vitro*. The results indicated that the ability of Taxol to induce apoptosis was enhanced following over-expression of Smac in the A549 NSCLC cell line.

Materials and Methods

Materials

Human lung cancer cell line A549 cells were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China). RPMI 1640 culture medium was from Gibco BRL (Carlsbad, USA). A549 cells were maintained in RPMI 1640 with 10% fetal bovine serum. TRIzol and Transfection kit were from Invitrogen (Carlsbad, USA) and the PCR kit was from Promega (Madison, USA). Primers were designed based on the gene sequence in GenBank:smac (NM_019887.3) and synthesized by Jierui Company (Shanghai, China) as follows: upstream 5′-GCTCTAGAATGGCGGCTCTGAAGAGTTGGCTGT-3′ (including *Xba*I restriction site), downstream 5′-GCGG ATCCTCAATCCTCACGCAGGT-3′ (including *Bam*HI restriction site). Restriction enzymes *Xba*I and *Bam*HI and T_4 DNA ligase were purchased from Takara (Dalian, China). Taxol (Qilu Pharmaceutical Factory, Jinan, China) was diluted to the appropriate concentrations in cell culture medium before being added to cells.

Reverse transcription–polymerase chain reaction and recombinant plasmid pcDNA3.1/Smac construction

Total RNA was extracted from human testis tissue in accordance with the TRIzol kit's instructions, and *Samc* cDNA was amplified with 0.1 μg RNA according to reverse transcription–polymerase chain reaction (RT–PCR) kit's instructions. The amplification reaction involved 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s, and 72°C for 1 min. PCR products were observed under 1.5% agarose gel electrophoresis and the target fragment was recovered (746 bp). The fragments were inserted into the *Xba*I and *Bam*HI sites of the pcDNA3.1 to construct the recombinant plasmid pcDNA3.1/Smac. The inserts were identified by agarose gel electrophoresis and sequencing.

Cell transfection

A549 cells were inoculated into six-well plates at a density of 106 cells per well, and transfected according to Lipofectamine 2000 instruction when the cell density reached 90%. The transfected cells were collected after 48 h. The expression of Smac was detected by RT-PCR and western blot.

Western blot analysis

A549 cells were collected after 24-h transfection and 24-h induction by Taxol. Cytoplasmic protein was extracted, and the protein concentration was determined using the Bradford method. The protein (100 μg) was added to each well for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride (PVDF) membrane for Ponceau red staining. The effect of a transfer film and the position of the target protein were determined according to the marker protein. The PVDF membrane was incubated with 5% bovine serum albumin at 37°C for 1 h, followed by incubation with mouse anti-human Smac monoclonal antibody (1 : 500) and then with horseradish peroxidase-labeled goat anti-mouse IgG antibody (Shanghai Mengsheng Biological Research Ltd, Shanghai, China) at 37°C for 1 h, respectively. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference. The signals were detected with an ECL chemiluminescence kit. The experiment in each group was repeated three times.

Invasive analysis

Twenty-four-well matrix gel invasion chambers (BD, Franklin Lakes, USA) were used. The size of membrane pore between the upper and lower chambers is 6 μm. The extracellular matrix gel was covered onto the upper chamber membrane surface, which can simulate the body extracellular matrix and the basement membrane environment. The transfected A549 cells were mixed with serum-free RPMI 1640 to the concentration of 2×10^5 cells/ml. The cell suspension (200 μl) was added to the upper chamber, and 500 μl of RPMI 1640 with 10% fetal calf serum was added to the lower chamber. After 48-h culture, the cells moved into the lower chamber were fixed with formaldehyde, and observed under the light microscope (×400). The cells in six fields were randomly counted and the mean value was calculated as the number of invasive cells.

Apoptosis assay

The cells were divided into six groups: untransfected control group, empty plasmid pcDNA3.1 group, pcDNA3.1/Smac group, Taxol group, empty plasmid pcDNA3.1 + Taxol group, and pcDNA3.1/Smac + Taxol group. Taxol (final concentration of 5 g/ml) was added to the medium at 12 h after transfection, and the cells were cultured for another 24 h. Then, the cells were collected and detected with Annexin V-FITC/PI by flow cytometry (FCM).

Cell cloning ability assay

Monolayer cultured cells in the logarithmic growth phase were dispersed into single cell suspension and counted. The pre-treated cells were seeded into six-well plates at 1000 cells per well, and cultured for 12 days. The culture medium was discarded and the cells were washed with PBS twice, then fixed with 5 ml ethanol for 15 min and stained with Kaji Muse dye for 10–30 min. The number of clones was counted (more than 50 cell mass is a clone). Plating efficiency (PE) = number of colony forming/seeded cells × 100%. Survival fraction (SF) = PE in treatment group/PE in control group.

Statistical analysis

The results were expressed as mean ± SD. One-way analysis of variance was used to determine the levels of difference between all groups. $P < 0.05$ was considered statistically significant.

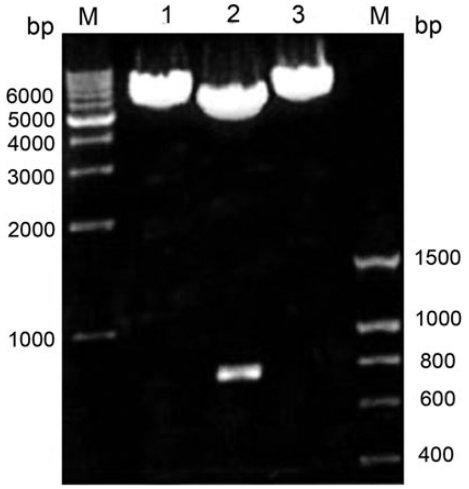

Figure 1. Identification of the recombinant plasmid pcDNA3.1/Smac
M, DNA marker; 1, pcDNA3.1/Smac of *Bam*HI enzyme digestion; 2, pcDNA3.1/Smac of double-enzyme digestion. 3, pcDNA3.1/Smac of *Xba*I enzyme digestion.

Results

Recombinant plasmid pcDNA3.1-Smac

The recombinant plasmid DNA was identified with the restriction enzyme. The agarose gel (10 g/l) electrophoresis showed that strip size was consistent with the expected design (746 bp) (**Fig. 1**). The sequence was the same as that in the Gene bank.

The expression of Smac in the transfected cells

To analyze the effect of Smac in the drug-treated lung cancer cells, we first determined the expression of Smac in transfected cells. RT-PCR and western blot analysis showed that pcDNA3.1/Smac transfected cells had a higher expression than the control cells (no transfected cells) at the mRNA level and protein level (**Fig. 2**). And the empty plasmid pcDNA3.1 transfected cells had no significant difference when compared with the control cells in the expression of Smac ($P > 0.05$).

Smac reduced A549 cell invasion

To investigate the invasive ability of the cells, we performed the invasion assay. The results showed that the number of transmembrane cells was significantly reduced in the experimental group (pcDNA3.1/Smac group) than in the control group (untransfected) ($P < 0.05$). The cell structure was not clear under a light microscope, and the invasion on the reconstituted basement membrane weakened (**Fig. 3**, $\times 400$).

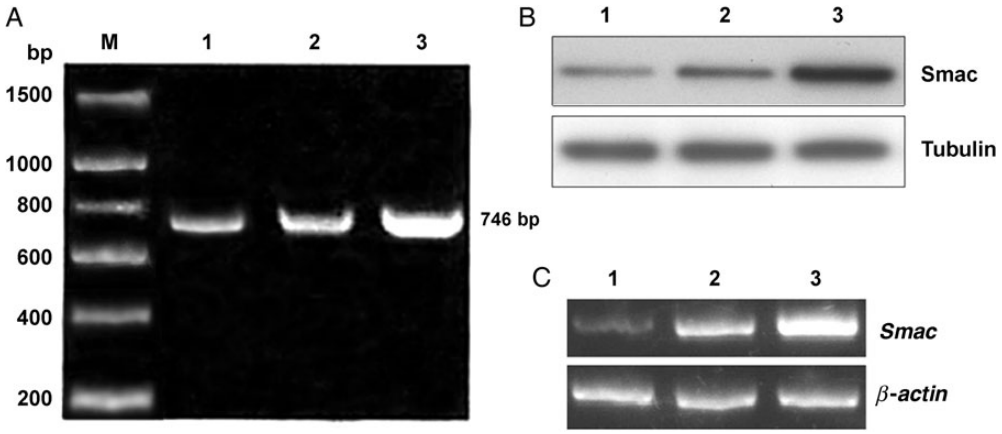

Figure 2. The expression of Smac in the transfected cells (A) RT–PCR amplification of Smac after transfection. (B) The expression of Smac protein in the different cells. (C) The expression of *Smac* mRNA in the different cells. M, DNA marker; 1, untransfected control group; 2, empty plasmid pcDNA3.1 group; 3, pcDNA3.1/Smac.

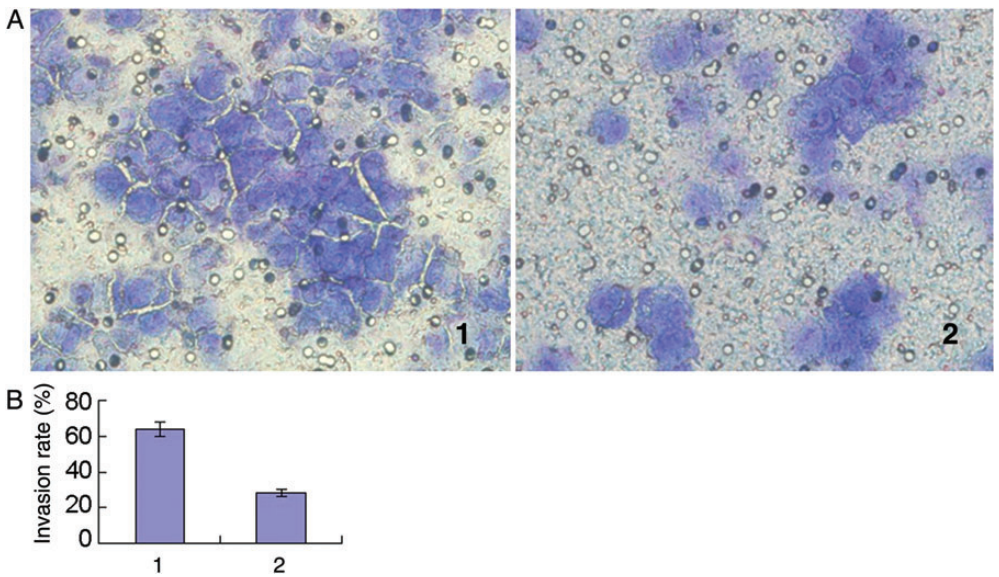

Figure 3. Cell invasion in the transfected cells (A) The representative pictures of the different groups ($\times 400$). (B) The quantification of the invasion cells. 1, untransfected control group; 2, pcDNA3.1/Smac group.

Smac increased cells apoptosis induced by Taxol

To investigate the induction of apoptosis in A549 cells under the treatment of Taxol, FCM apoptosis analysis was applied in different groups. The results showed that Smac over-expression induced a little apoptosis, but the difference was significantly compared with the empty vector group and the untransfected control group ($P < 0.05$). When the cells were treated with Taxol, the cells showed a higher apoptosis rate, particularly, the cell apoptosis rate was significantly increased in the pcDNA3.1/Smac + Taxol group when compared with that in the Taxol group and the pcDNA3.1/Smac group ($P < 0.05$). But the difference between the Taxol group and the empty plasmid pcDNA3.1 + Taxol group was not significant ($P > 0.05$) (**Table 1**, **Fig. 4**), which indicated that Smac over-expression increased the apoptosis rate of A549 cells under the treatment of Taxol.

Table 1. Apoptotic rate in different groups

Group	Apoptotic rate
Untransfected control group	1.47 ± 0.11
Empty plasmid PcDNA3.1 group	1.87 ± 0.12
pcDNA3.1/Smac group	3.19 ± 0.30*
Taxol group	10.69 ± 0.78
Empty plasmid PcDNA3.1 + Taxol group	12.03 ± 1.28
pc DNA3.1/Smac + Taxol group	20.26 ± 1.22**

*$P < 0.05$ vs. empty vector group and untransfected control group.
**$P < 0.05$ vs. Taxol group and pcDNA3.1/Smac group.

Smac inhibited A549 cell cloning ability

Cell proliferation may reflect its biological behavior. We examined the cell clone by plate assay. Results showed that cells' cloning ability in the experimental group was worse than that of other groups. There is statistical difference ($P < 0.05$) between these two groups. Observed visually, cell mass formed in relatively small quantities and sparse location (**Table 2**, **Fig. 5**).

Discussion

Many patients with lung cancer have already lost the chance of operation when diagnosed, chemotherapy become the main treatment for advanced lung cancer. Chemotherapy drug resistance is one of the important reasons of low treatment efficiency currently. So how to improve chemosensitivity and reverse drug resistance in lung cancer treatment is an urgent issue. The discovery of Smac and the elucidation of its structure and function have led to the rapid

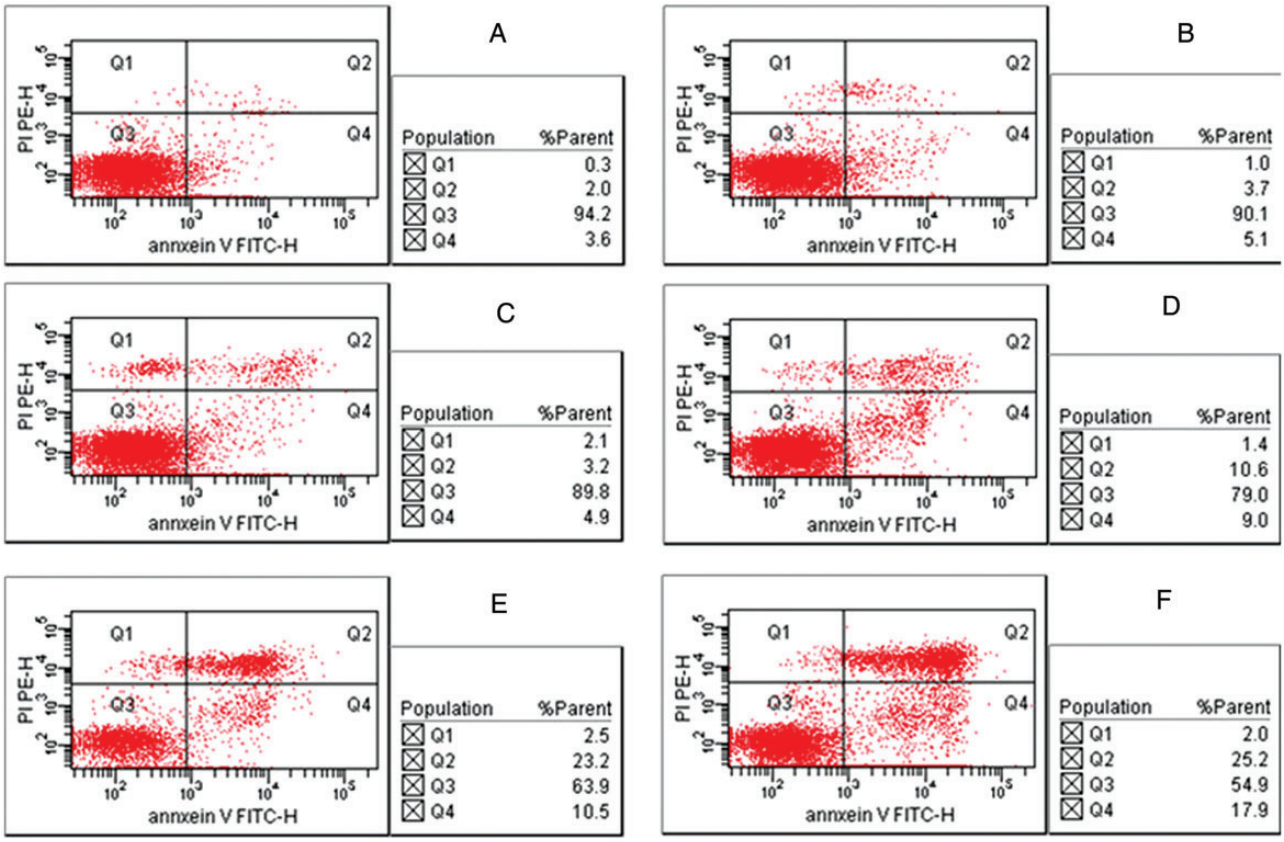

Figure 4. Cell apoptosis in different groups (A) Untransfected control group. (B) Empty plasmid pcDNA3.1 group. (C) pcDNA3.1/Smac group. (D) Taxol group. (E) Empty plasmid pcDNA3.1 + Taxol group. (F) pcDNA3.1/Smac + Taxol group.

development of Smac mimetics [10–12]. As a pro-apoptotic gene, Smac was discovered in 2000 by Du *et al*. [13] and Verhagen *et al*. [14]. Smac plays an important role in apoptosis through the mitochondrial pathway and death receptor pathway. Mature Smac (pure dimmer) locates in the membranes' cavity of mitochondria. When Smac is subject to apoptosis-inducing factors such as anti-cancer drugs, DNA damage, and ultraviolet radiation, the signal peptide is removed to form the active Smac [15,16]. Furthermore, the active Smac and cytochrome c are released from the mitochondria to the cytoplasm, and promote cell apoptosis by relieving the IAPs inhibition on caspase-9 and caspase-3 [17]. Over-expression of IAPs in tumor cells is the main reason to help tumor cells to evade immune surveillance, and XIAP is a leading member of the IAPs. Smac protein is an endogenous XIAP inhibitor, with stimulation of apoptosis signaling and release of cytochrome c from the mitochondria to the cytoplasm. Smac can combine with groove on the surface of XIAP-BIR3 by replacing caspase-9 so as to reverse XIAP inhibition on caspase-9, thereby releasing caspase-9, and then activating caspase-3 and amplifying caspase cascade apoptotic activity [18,19]. Therefore, inhibition of XIAP can induce lung cancer cells with high expression of XIAP apoptosis and promote tumor cells' sensitivity to the drug [20].

Taxol is one of the most widely used chemotherapeutics and is a first-line drug for lung cancer treatment. However, high doses of Taxol produce strong side effects in patients, such as severe allergic reactions, myelosuppression and neurotoxicity, and low doses do not effectively curtail tumor growth. Therefore, the advanced Taxol treatments should improve its anti-tumor effect at lower doses to reduce toxicity at high dose. *Smac* gene is highly expressed in many normal tissues, and it shows a lower expression in tumor

Table 2. Cloning ability in different groups

Group	Mean ± SD	PF	SF
Untransfected control group	165 ± 8	0.165	1.000
Empty plasmid PcDNA3.1 group	102 ± 4	0.102	0.617
pcDNA3.1/Smac group	116 ± 6	0.116	0.700
Taxol group	106 ± 6	0.106	0.643
Empty plasmid PcDNA3.1 + Taxol group	110 ± 6	0.110	0.665
pc DNA3.1/Smac + Taxol group	54 ± 7	0.054	0.329

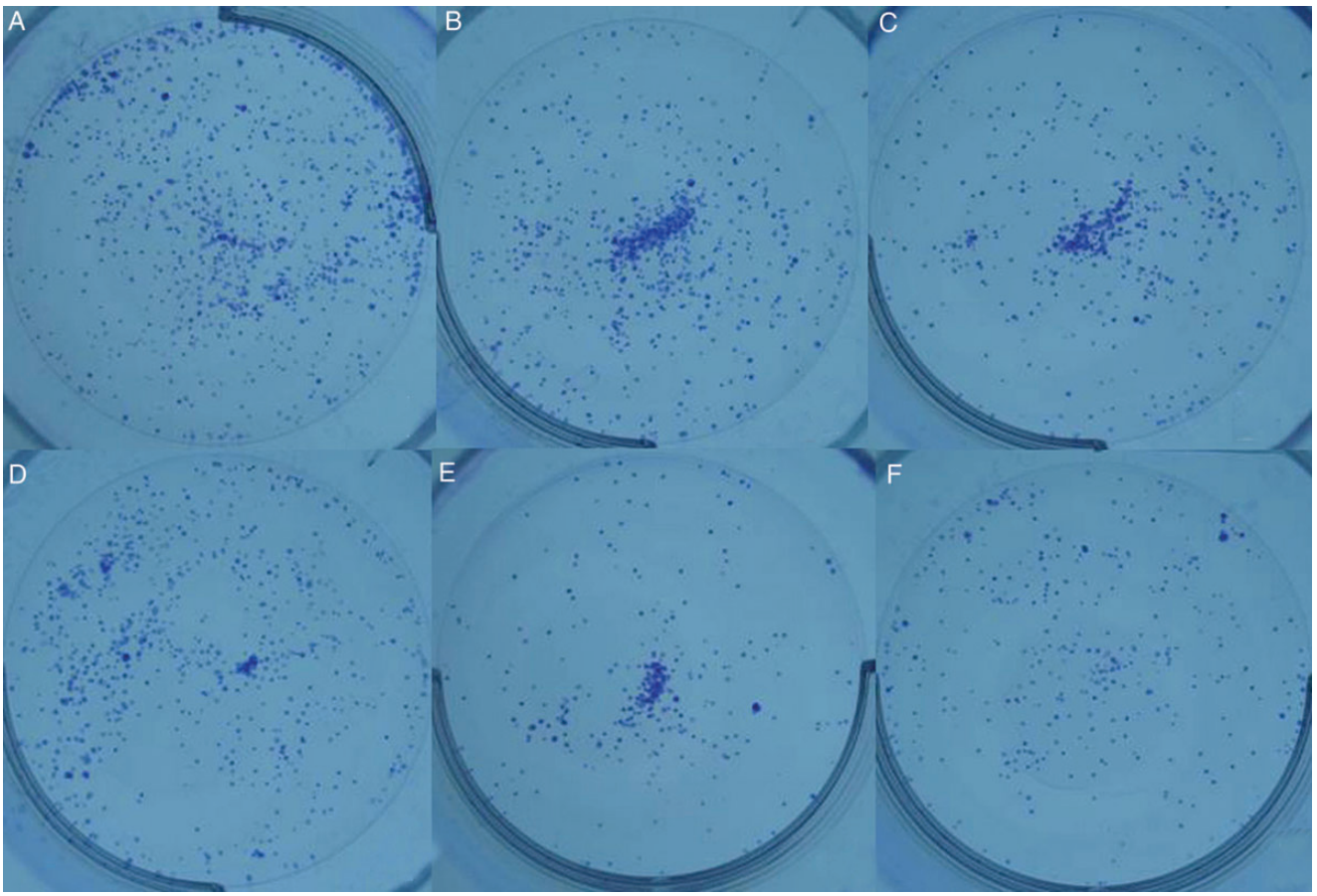

Figure 5. Clone formation analysis and the clone number of A549 cells in different groups (A) Untransfected control group (791). (B) Empty plasmid pcDNA3.1 group (529). (C) pcDNA3.1/Smac group (386). (D) Taxol group (618). (E) Empty plasmid pcDNA3.1 + Taxol group (236). (F) pcDNA3.1/Smac + Taxol group (352).

tissue. Smac is released into the cytosol in response to many apoptotic stimuli, such as chemotherapy and radiotherapy [14]. Previous studies have shown that over-expression of Smac can induce apoptosis in ovarian cancer cell lines. [21,22], which indicated that increase of the cytosolic Smac protein might be a potential approach for cancer therapy.

In this study, the gene of *Smac* was obtained from human testis tissue and cloned into the expression vector pcDNA3.1. The recombinant expression plasmid pcDNA3.1/Smac was transfected into human lung cancer cells A549 using lipofectamine. We found that Smac expression was higher than that in the control group. However, the number of invasive cells and invasive ability decreased obviously, which suggested that high expression of Smac can reduce the invasive ability of lung cancer cell. These results are similar to the previous reports that have been done in other types of malignant tumors. Apoptosis was significantly higher than that in other groups when Smac acted on the lung cancer cells A549 induced by Taxol. When the Smac over-expressing A549 cells were treated with Taxol, the apoptosis rate was significantly increased when compared with that in the control cells treated with Taxol, and the cells cloning ability decreased obviously, which indicated that high expression of Smac can significantly enhance apoptosis in A549 cells, improve A549 cell sensitivity to Taxol, and increase the cytotoxic effect of Taxol. So Smac and Taxol may play a synergistic anti-tumor effect, which will provide theoretical and experimental basis for biological treatment of lung cancer. Smac mRNA expression has been reported to be significantly lower in lung cancer tissue than in normal lung tissue, and NSCLC patients with higher Smac mRNA expression had significantly longer progression-free survival and overall survival with adjuvant chemotherapy [8], which is consistent with our results.

In summary, we have demonstrated that over-expression of Smac increases the sensitivity of lung cancer A549 cells to Taxol treatment, and transfection of Smac to tumor cells represents one potential therapy. However, further study is required to precisely identify the effects of Smac in lung cancer models and to determine its synergy in combination with chemotherapy.

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