

Original Article

Effect of Second mitochondria-derived activator of caspase in combination with Docetaxel on lung cancer cell A549

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Received January 29, 2015; Accepted April 21, 2015; Epub May 15, 2015; Published May 30, 2015

Abstract: This study was to investigate inhibiting effect of structurally unique Second mitochondria-derived activator of caspase (Smac) in combination with Docetaxel on lung cancer cell line A549. Results showed that the expression of Smac in transfected A549 cells was higher than the control cells both at mRNA level and protein level ($P < 0.05$). Smac over-expression induced a little apoptosis, however, when treated with Docetaxel together, the cells showed a higher apoptosis rate. The apoptosis rate was significantly increased in Smac + Docetaxel group when compared with that in Smac group and Docetaxel group ($P < 0.05$). Cells cloning ability in Smac + Docetaxel group was worse than that of other groups ($P < 0.05$), cell mass formed in relatively small quantities and sparse location. Thus, over-expression of Smac increases the sensitivity of lung cancer A549 cells to Docetaxel treatment, and transfection of Smac to tumor cells might provide a potential therapy modality.

Keywords: Second mitochondria-derived activator of caspase, lung cancer, Docetaxel, apoptosis, cloning ability

Introduction

Non-small cell Lung cancer (NSCLC) is a common and highly malignant respiratory cancer, and can be treated by surgery with chemotherapy as auxiliary treatment. Data indicated that approximately 80% of NSCLC patients are inoperable at diagnosis [1]. The reason why the incidence of lung cancer and fatality rate is high is that normal chemotherapy is not effective to prevent the growth of tumor. Inhibition of tumor development and progression was proved to significantly improve the therapeutic outcomes and patients' survival and life quality. Ways to improve tumor response to existing chemotherapies without affecting normal cells would be a major step forward.

The growth, metastasis and relapse of lung cancer are apoptosis-dependent. Therefore, it becomes an important direction to cancer treatment by inducing tumor apoptosis and enhancing chemosensitivity in recent years [2].

Inhibitors of apoptosis proteins (IAP) are key regulators of apoptosis and are inhibited by Smac. The unique function of Smac is that it sensitizes cells to apoptosis induced by apoptotic stimuli and therefore has therapeutic potential [3]. Studies showed that NSCLC patients with higher Smac mRNA expression had significantly longer progression-free survival and overall survival with adjuvant chemotherapy [4, 5]. In our study, we investigated the role of Smac in apoptosis of lung cancer cells A549 induced by Docetaxel.

Materials and methods

Cell lines and culture

Human lung cancer cell line A-549 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences and maintained in RPMI-1640 (Gibco USA) with 10% fetal bovine serum. Cells in logarithmic growth phase were used for experiments. Docetaxel was from Qilu

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Company in China and diluted to the appropriate concentrations in cell culture medium before being added to cells. Transfected A549 cells and plasmid pcDNA3.1 were kindly provided by Dr. Cong from Shandong University.

RT-PCR and Western blot analysis

Total RNA was extracted from transfected A549 cells in accordance with the TRIzol kit's instructions, and RT-PCR reaction was conducted to examine Smac mRNA expression. The cells were divided into three groups: untransfected control group, empty plasmid pcDNA3.1 group and pcDNA3.1/Smac group. The primer sequences of Smac were as follows: upstream primer 5'-GGAGCCAGAGCTGAGAYGAC-3'; downstream primer 5'-CTCTGACCCAGGTAGGCAA A-3', β -actin was used as an internal control. The results were analyzed by 20 g/L agarose gel electrophoresis and photographed by Bio-Rad gel imaging system.

Cytoplasmic protein of the transfected cells was extracted after 24 h induction by Docetaxel. The protein was added to each well for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride 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 polyvinylidene difluoride 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 at 37°C for 1 h, respectively. Glyceraldehyde-3-phosphate dehydrogenase with an ECL chemiluminescence kit. The expression in each group was repeated three times.

Cells apoptosis assay

Six groups were divided: untransfected control group, empty plasmid PcDNA3.1 group, pcDNA3.1/Smac group, Docetaxel group, empty plasmid PcDNA3.1 + Docetaxel group and pcDNA3.1/Smac + Docetaxel group. Docetaxel was added (final concentration of 5 g/ml) and the cells were cultured for 24 hours. Annexin V-FITC dye 2 mL and PI dye 5 ml were added after the cells were collected. Test was repeated three times by Flow Cytometry.

A549 cell cloning ability by plate assay

Monolayer culture cells in logarithmic growth phase were dispersed into single cell suspension and counted. The pre-treated cells were seeded into 6-well plates at 1000 per well, and cultured for 12 days. The culture medium was discarded and cells were washed with PBS twice, then fixed with 5 ml ethanol for 15 min and dyed with Kaji Muse dye for 10-30 min. The number of clones as counted (more than 50 cell mass is a clone). Plating efficiency (PE) = number of colony 2 forming/seeded cells \times 100%. Survival fraction (SF) = PE in treatment group/PE in control group.

Statistics

SPSS20.0 is used for statistical analysis. Data are expressed as means \pm standard deviation. Paired t-test was used to compare Smac activity in A549 cells treated with or without Docetaxel. One-way ANOVA with a Dunnett Multiple Comparison Test was used to determine the levels of difference between all groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

Smac expression in transfected cells

The expression of Smac in transfected A549 cells was determined firstly to analyze the effect of Smac in the drug-treated lung cancer cells. Results of RT-PCR and Western blot analysis showed that the transfected cells had a higher expression than the control group at mRNA level and protein level ($P < 0.05$) (**Figure 1**). There was no significant difference between the control group and empty plasmid pcDNA3.1 group ($P > 0.05$).

Cells apoptosis induced by Docetaxel increased by Smac

FCM apoptosis analysis showed that transfection pcDNA 3.1/Smac only induced less apoptosis, but the difference was significant compared to empty vector group and the untransfected control group ($\bar{x} \pm s$, $P < 0.05$). The rate of cell apoptosis was significantly higher in pcDNA3.1/Smac + Docetaxel group (20.26 ± 1.22) than that in Docetaxel group (10.69 ± 0.78) and pcDNA3.1/Smac group (3.19 ± 0.30). Deference was not found between Docetaxel

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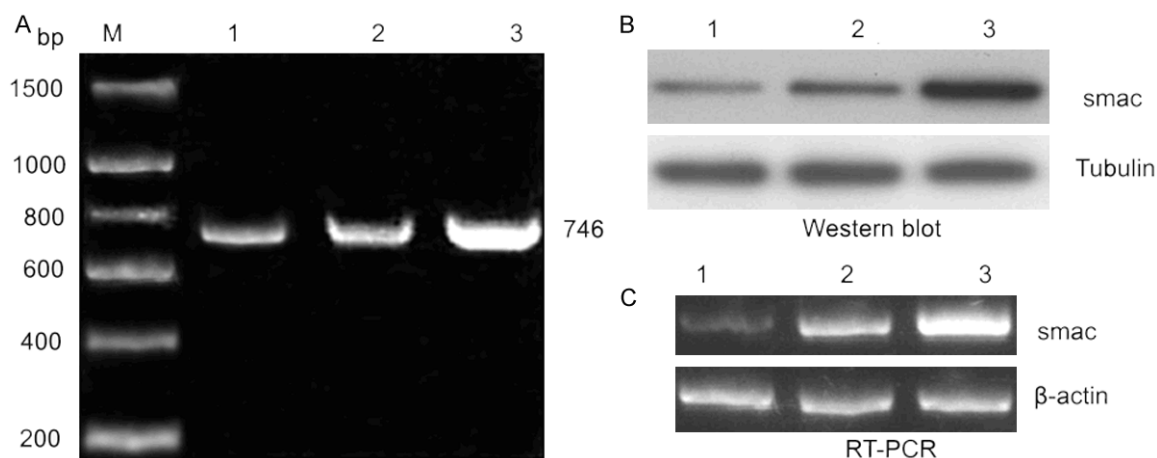


Figure 1. Smac transcription in the mRNA level in different groups. M: DNA marker; 1: untransfected control group; 2: empty plasmid PcDNA3.1 group; 3: pcDNA3.1/Smac + Taxol group.

Table 1. Apoptotic rate in different groups

Group	Apoptotic rate
Control group	1.47±0.11
Empty plasmid pcDNA 3.1 group	1.87±0.12
pcDNA 3.1/Smac group	3.19±0.30*
Docetaxel group	10.69±0.78
Empty plasmid pcDNA 3.1+ Docetaxel group	12.03±1.28
pcDNA 3.1/Smac + Docetaxel group	20.26±1.22**

* $P < 0.05$ vs. empty vector group and untransfected control group.

** $P < 0.05$ vs. Docetaxel group and pcDNA 3.1/Smac group.

group (10.69±0.78) and empty plasmid pcDNA3.1 + Docetaxel group (12.03±1.28) ($P > 0.05$) (Table 1; Figure 2).

Cell cloning ability in different groups

Cell proliferation may reflect its biological behavior. We examined the cell clone by plate assay. It showed that PE in pcDNA3.1/Smac + Docetaxel group was worse than that of Docetaxel group (0.0548 vs 0.106), and the SF in the two groups was 0.329 vs 0.634, respectively. There is statistical difference ($P < 0.05$). Observed visually, cell mass formed in relatively small quantities and sparse location (Figure 3; Table 2).

Discussion

Lung cancer is the leading cause of cancer deaths in males and the second in females globally. Despite the advances in early detection and standardized treatment regimens, 5-year survival rates for lung cancer are still

relatively poor. Typically, lung cancer is at an advanced stage at the time of diagnosis, and treatment is usually not very effective [5]. Approximately 80% of lung cancer patients are inoperable at diagnosis, and normal chemotherapy is unable to effectively prevent the growth of the tumor. Therefore, molecular techniques for choosing therapeutic methods need to be improved for better treatment. The recent discovery of Smac and the elucidation of its structure and function have led to the rapid development [6-8], comprising Smac derivative and mimicking molecules. Smac is an endogenous proapoptotic protein that resides in the mitochondria and is released when a cell is triggered to undergo programmed cell death.

Smac (also known as DIABLO) was initially discovered independently by two groups in 2000 [9, 10]. The human gene is located on chromosome 12p and is composed of seven exons. The 1.5 kb cDNA of Smac encoding 239 amino acids produces a protein of 27 kDa. One of the mechanisms by which Smac promotes apoptosis is through its ability to inhibit IAPs, by direct inhibition and/or proteasomal degradation of some members of the IAP family. Overexpression of IAPs in tumor cells is the main reason to help tumor cells evade immune surveillance, and XIAP (X linked inhibitor of apoptosis protein) is a leading member of the IAPs. Smac protein is endogenous XIAP inhibitor, with stimulation of apoptosis signaling and release of cytochrome C from mitochondria to the cytoplasm. Smac

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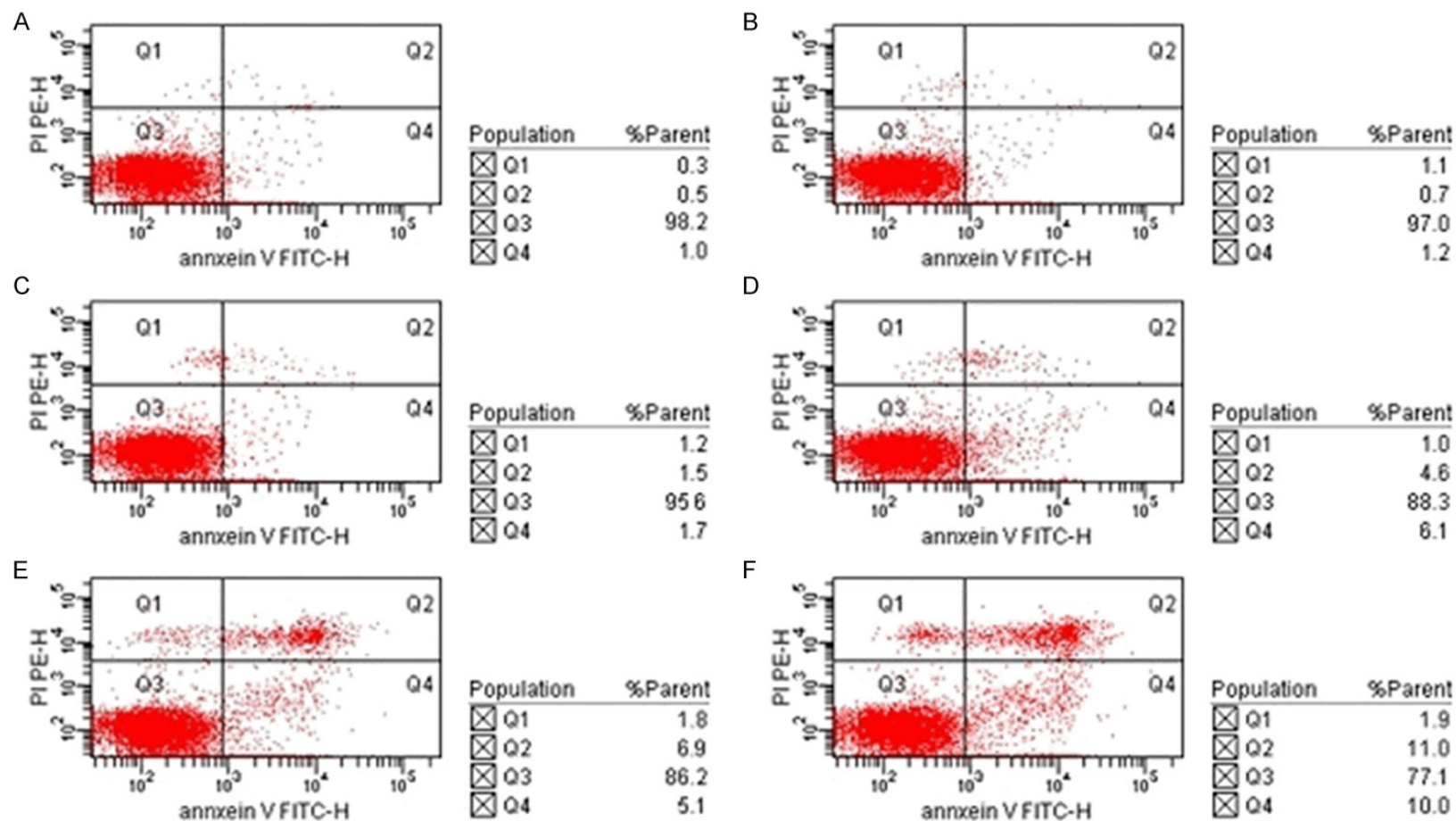


Figure 2. Flow Cytometry Diagram of apoptosis in different groups. A: Control group; B: Empty plasmid pcDNA 3.1 group; C: pcDNA 3.1/Smac group; D: Docetaxel group; E: Empty plasmid pcDNA 3.1+ Docetaxel group; F: pcDNA 3.1/Smac + Docetaxel group.

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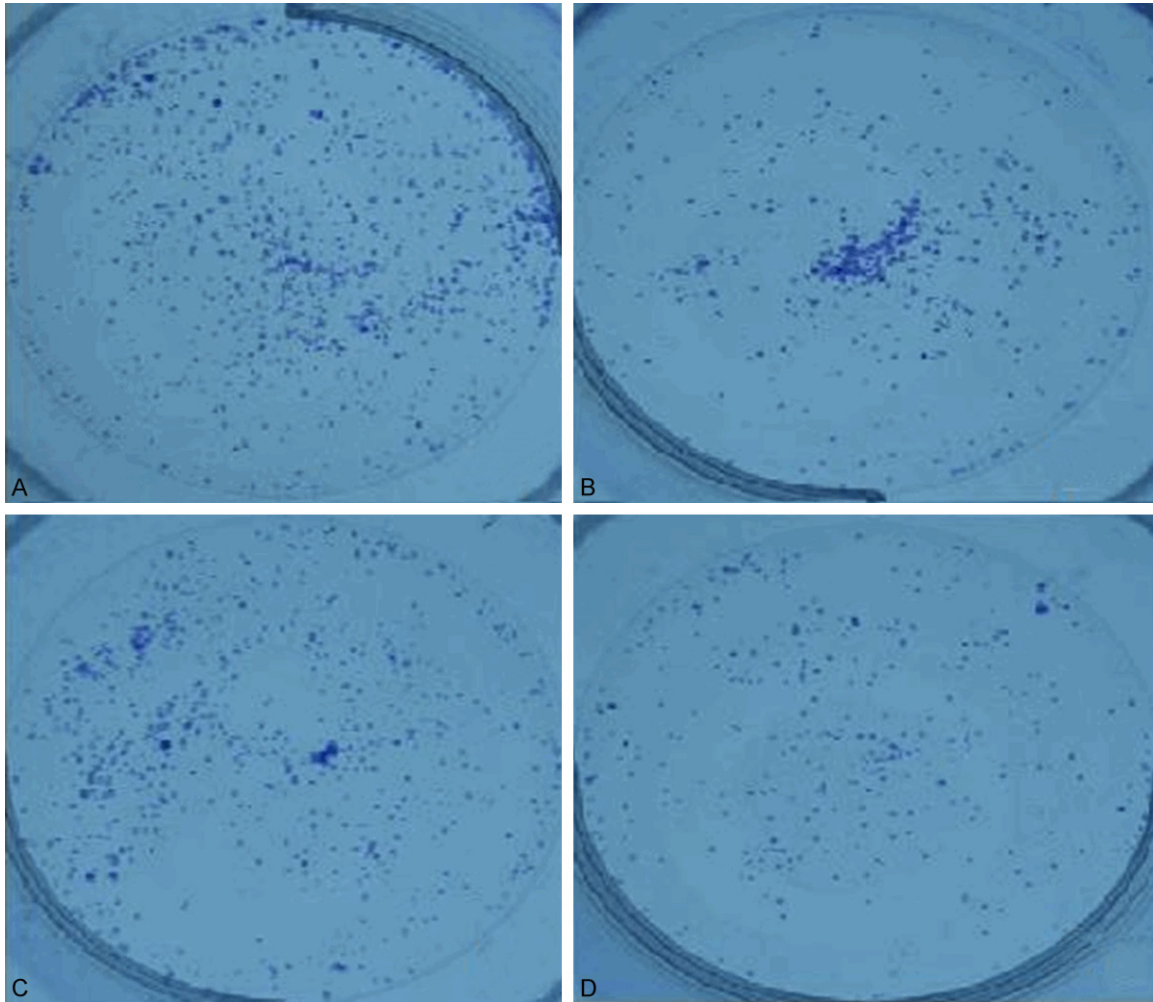


Figure 3. The cell clone was examined by plate assay in different group. A: Control group; B: pcDNA3.1/Smac group; C: Docetaxel group; D: pcDNA3.1/Smac + Docetaxel group.

Table 2. Cloning ability in different groups

Group	Mean \pm SD	PE	SF
Control group	165 \pm 8	0.165	1.000
pcDNA3.1/Smac group	116 \pm 6	0.116	0.700
Docetaxel group	106 \pm 6	0.106	0.634
pcDNA3.1/Smac + Docetaxel group	54 \pm 7*	0.0548*	0.329*

Note: Plating efficiency (PE) = number of colony 2 forming/seeded cells \times 100%; Survival fraction (SF) = PE in treatment group/PE in control group.
* $P < 0.05$.

can combine with groove on surface of XIAP-BIR3 by replacing caspase-9 so as to reverse XIAP inhibition on caspase-9, thereby releasing caspase-9, and then activate the caspase-3 and amplify caspase cascade apoptotic activity [11]. Therefore, inhibition of XIAP can induce lung cancer cell with high expression of XIAP apoptosis and promote tumor cells sensitivity to the drug [12].

More recently researches discovered that Smac displays differential expression between normal and cancer tissues [13], with expression lost in tumor [14], suggesting that Smac is an anti-cancer or tumor suppressor gene. NSCLC patients with higher Smac mRNA expression had significantly longer progression-free survival and overall survival with adjuvant chemotherapy [15]. In our study, the transfected cells have been proven high expression of Smac both at mRNA level and protein level compared to control group. Rate of cell apoptosis was significant higher in pcDNA3.1/Smac + Docetaxel group than that in Docetaxel group and pcDNA3.1/Smac group. It indicated that over-expression of Smac significantly sensitized A549 cells to chemotherapy. These results are

similar to the previous reports that have been done in other types of malignant tumors [16]. Docetaxel is widely used chemotherapeutics and is a first-line drug for lung cancer treatment. However, high doses produce strong side effects in patients, such as myelosuppression, severe allergic reactions and neurotoxicity, and low doses do not effectively curtail tumor growth. Our study showed that both Docetaxel and overexpression of Smac were able to promote A549 cells apoptosis. However, their combination will greatly enhance the rate of apoptosis. It indicated that Docetaxel treatment could improve its anti-tumor effect at lower doses with overexpression of Smac.

In summary, increased expression of cytosolic mature Smac can be used as an effective regulatory signal to promote chemotherapy-induced apoptosis and enhance chemosensitivity of lung cancer cells. It is clear that Smac have great therapeutic potential for the treatment of lung cancer patients. Further study is required to precisely identify the effects of Smac in lung cancer models and its synergy in combination with chemotherapy.

Acknowledgements

This research was supported by the Science and Technology research funded projects of Ministry of Education of the People's Republic of China (20110131120062) and the Development Projects of Science and Technology of Shandong Province, China (2012GGE27073).

Disclosure of conflict of interest

None.

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