Original Article Effect of Smac in combination with cisplatin on esophageal cancer cell line ECA109

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Abstract: Objective: This study was to investigate inhibiting effect of structurally unique Second mitochondria-derived activator of caspase (Smac) in combination with cisplatin on esophageal cancer cell line ECA109. Methods: PcDNA3.1-Smac (ECA109/Smac group), pcDNA3.1 (ECA109/neo group) and PBS (ECA109 or control group) were transfected into ECA109 cells respectively, and transfected cells which expressed Smac stably were got. Smac protein expression was analyzed by Western blot. The invasive ability of cells was examined. Flow cytometry was used to analyze apoptosis induced by cisplatin with Annexin V/PI double staining technique. Results: Smac gene was successfully transfected into ECA109 cell, over-expression of Smac could decrease cell invasive ability obviously compared to control group (P<0.05). Apoptosis rate of cells induced by cisplatin in ECA109/Smac group was significant higher than that in ECA109/neo and ECA109 group (P<0.05). Conclusion: It indicated that over-expression of Smac increases the sensitivity of esophageal cancer ECA109 cells to cisplatin treatment, combination of conventional anticancer drug with Smac may be beneficial for the treatment of esophageal cancer.

Keywords: Second mitochondria-derived activator of caspase, esophageal cancer, cisplatin, invasion, apoptosis

Introduction

Esophageal cancer is a common and highly malignant gastrointestinal cancer. Esophageal squamous cell carcinoma (ESCC) is the fourth most prevalent malignancy in China [1]. ESCC can be treated by surgery with chemoradiotherapy as auxiliary treatment. However, the outcome is not ideal with low 5-year survival rate ranging from 20 to 35% [2]. Concurrent chemo/ radiotherapy is the standard treatment for patients with advanced diseases. Cisplatin, induce apoptosis by DNA adducts, is often used to treat ESCC. However, resistance to apoptosis certainly plays a very relevant role in tumor development. Smac/DIABLO is an important new apoptosis regulating genes which was discovered in 2000 [3]. Many research confirmed that over-expression of Smac gene can significantly induce tumor apoptosis by enhancing chemosensitivity and radiosensitivity, and provided a new way for cancer treatment. Few literatures have been reported about Smac gene expression in ESCC. In this study, we constructed a stable esophageal cell lines with over-expression of Smac and investigated the role of Smac in apoptosis of ESCC induced by cisplatin.

Materials and methods

Cell lines and culture

Human ESCC cell line ECA109 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences and maintained in RPMI-1640 (Gibico USA) with 10% fetal bovine serum. Cells in logarithmic growth phase were used for experiments. Cisplatin was from Qilu Pharmaceutical Company in China and diluted to the appropriate concentrations in cell culture medium before being added to cells. Recombinant pcDNA3.1-Smac and empty vector pcDNA3.1 were kindly provided by Dr. Cong from Shandong University. Lipofectamine [™] 2000 was purchased from Invitrogen Corporation. Rabbit anti-human Smac monoclonal antibody, goat anti-rabbit IgG antibody labeled with horseradish peroxidase were from Santa Cruz Company in 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'-GCTCTAGAATGGG CGGGCTCTGA-AGAGTTGGCTGT-3' (including Xba I restriction site), downstream 5'-GCGG ATCCTCAATCCTC-ACGCAGGT-3' (including *BamH* I restriction site). Cisplatin was diluted to the appropriate concentrations in cell culture medium before being added to cells.

Gene transfection and cell lines selection

The six-well plates were added pcDNA3.1-Smac (ECA109/Smac group), pcDNA3.1 (ECA109/ neo group) and PBS (ECA109 or control group) respectively. ECA109 cells were inocula 5 s ted into six-well plates at a density of 10^6 cells per well, and transfected according to Lipofectamine TM 2000 instruction when the cell density reached 90%. The transfected cells were collected after 48 h for selection with G418. Firstly, 0.8 g/L G418 was used for 2 weeks, then 0.2 g/L G418 for six weeks. Transfection efficiency was observed under the fluorescence microscope.

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 μ g RNA according to 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 °C for 1 min. PCR products were observed under 1.5% agarose gel electrophoresis and the target fragment was recovered (746 bp). The fragment was inserted into *Xba* I and *BamH* I sites of the pcDNA3.1 to construct the recombinant plasmid pcDNA3.1-Smac. The inserts were identified by agarose gel electrophoresis and sequencing.

Western blot analysis

Referring to "Manual of Molecular Cloning Laboratory", the transfected cells were collected and cytoplasmic protein was extracted. The protein concentration was determined using the Bradford method. The protein ($100 \mu g$) 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 PVDF membrane was incubated with 5% bovine serum albumin at 37°C for 1 h, followed by incubation with rabbit anti-human monoclonal antibody (Smac 1:500, β -actin 1:1000) and then with goat anti-rabbit IgG antibody (1:500) at 37°C for 1 h, respectively. Glyceraldehyde-3phosphate 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

24-well matrix matrix gel invasion chambers 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 ECA109 cell were mixed with serum-free RPMI 1640 to the concentration of 2×10⁵ 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. 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 two group: cisplatin treated group and untreated group. Each group included ECA109/Smac, ECA109/neo, ECA109 three groups. The cells were added into 6-well $(0.3 \times 10^6 \text{ per well})$ and cultured for 48 h. In cisplatin treated group, different final concentration of cisplatin (1 mg/L, 5 mg/L, 10 mg/L) was added according to plasma drug concentration, and were cultured for another 24 h. Then, the cells were collected and detected with Annexin V-FITC/PI by flow cytometry (FCM). The experiment in each group was repeated three times.

Statistics

SPSS17. O software was used for data processing. Count data was expressed as $\overline{x} \pm s$, P<0.05 was considered statistically significant. Two



Figure 1. The expression of Smac in the transfected cells A: ECA109/neo group, B: ECA109 group, C: ECA109/Smac group.



Figure 2. Smac protein expression in different group by Western blot analysis. A: ECA109/neo group, B: ECA109 group, C: ECA109/Smac group.

independent samples were compared with *t* test, and diverse samples with variance analysis, data were tested with homogeneity of variance test.

Results

Transfection efficiency

Transfection efficiency was observed after G418 selection under a fluorescence microscope. Transfected cells (ECA109/Smac) which expressed Smac stably were got. The growth of cells was exuberant with normal morphology. There was no difference in cell morphology compare to ECA109 cell.

Recombinant plasmid pcDNA3.1-Smac

The recombinant plasmid DNA was identified with the restriction enzyme. The agarose gel electrophoresis showed that strip size was consistent with the expected design (**Figure 1**). The sequence was the same as that in the Gene bank.

Smac protein expression

Bands appeared in the expected relative molecular mass (*Mr*) 42000 and 27000 (**Figure 2**). Grayscale ratio analysis showed $0.8321\pm$ 0.023, 0.1523±0.0009 and 0.1399±0.114 in ECA109/Smac, ECA109/neo and ECA109 group respectively. The ratio in ECA109/Smac group was 5.46 times to that in ECA109/neo group and 5.95 times to ECA109 group, the difference was significant (*P*<0.05). There was no difference between ECA109/neo and ECA109 group (*P*>0.05).

Smac reduced ECA109 cell invasion

The results showed that the number of transmembrane cells was significantly reduced in ECA109/Smac group than in the ECA109/neo group and ECA109 group (P<0.05). The cell structure was not clear under a light microscope, and the invasion on the reconstituted basement membrane weaken (**Figure 3**).

Cells apoptosis induced by cisplatin increased by Smac

In cisplatin untreated group, apoptosis rate in ECA109/Smac, ECA109/neo and ECA109 group was (10.5 ± 1.1) %, (9.3 ± 0.8) % and $(9.4\pm$ 1.0)% respectively. No significant difference was found between them (*P*>0.05). In cisplatin treated group, apoptosis rate in ECA109/Smac group was significant higher than that in ECA109/neo and ECA109 group (*P*<0.05), and cell apoptosis rate increased with the cisplatin concentration. There was no difference between ECA109/neo and ECA109 group (*P*>0.05) (**Table 1**).

Discussion

Approximately half of patients lost the chance of operation when diagnosed, chemotherapy or radiotherapy become the main treatment for advanced esophageal cancer. Cisplatin is often preferred to treat ESCC patients as conventional chemotherapeutics [4]. However, there is no convincing evidence for significant improvement in the overall survival rate. Chemotherapy drug resistance is one of the important reasons of low treatment efficiency currently. So how to improve chemosensitivity and reverse drug resistance in ESCC treatment is an urgent issue. The discovery of Smac and the elucidation of its structure and function have led to the rapid development [5, 6]. However, the role of Smac in ESCC remains unclear.

Smac was initially discovered independently by two groups in 2000 [3]. The human gene is

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Figure 3. Invasion in ECA109/Smac group (A), ECA109/neo group (B) and ECA109 group (C) (×400).

located on chromosome 12p and is composed of seven exons. The 1.5 kb cDNA of Smac encodes 239 amino acids produce 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. Over-expression 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 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 [7]. Therefore, inhibition of XIAP can induce ESCC with high expression of XIAP apoptosis and promote tumor cells sensitivity to the drug [8].

More recently researches discovered that Samc displays differential expression between normal and cancer tissues [9], with expression lost in tumor [10], suggesting that Smac is an anti-cancer or tumor suppressor gene. Yang Xu et al [11] found that only 36.8% in ESCC tissues expressed Smac, compared 64.7% in adjacent normal tissues. Thus, Smac downregulation may contribute to esophageal carcinogenesis. Reduced Smac expression has also been

Table 1. Different apoptosis rate in cisplatin treate
group (%, $\overline{x} \pm s$)

Group	1 mg/L	5 mg/L	10 mg/L
ECA109/Smac	20.97±1.87	35.53± 2.21⁵	51.53±3.11℃
ECA109/neo	9.63±2.11ª	21.97±2.01ª	31.12±2.76ª
ECA109	9.31±1.98ª	20.93±1.87ª	28.26±2.98ª

°P<0.05 vs ECA109/Smac group; °P<0.05 vs 1 mg/L; °P<0.05 vs 1 mg/L.

described in other types of cancer [12, 13]. Importantly, our data revealed a significant association between Smac expression and chemotherapeutic response in ECA109 cells. In our study, the recombinant expression plasmid pcDNA3.1/Smac was successfully transfected into ECA109 cell using lipofectamine. The results showed that not only Smac expression was high in transfected cells, but its function was normal. Exogenous Smac gene could decrease invasive ability and increase apoptosis rate of ECA109 cells induced by cisplatin. It indicated that high expression of Smac can significantly improve ECA109 cell sensitivity to cisplatin, and increase the cytotoxic effect of cisplatin. These results are similar to the previous reports that have been done in other types of malignant tumors [14, 15]. So Smac and cisplatin may play a synergistic anti-tumor effect, which will provide theoretical and experimental basis for biological treatment of ESCC.

In summary, we have demonstrated that overexpression of Smac increases the sensitivity of esophageal cancer ECA109 cells to cisplatin treatment, and transfection of Smac to tumor cells represents one potential therapy. Further studies of Smac and other apoptosis regulators may help develop novel therapeutic strategies for esophageal cancer.

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Disclosure of conflict of interest

None.

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References

- [1] Umar SB, Fleischer DE. Esophageal cancer: epidemiology, pathogenesis and prevention. Nat Clin Pract Gastroenterol Hepatol 2008; 5: 517-526.
- [2] Purwar P, Bambarkar S, Jiwnani S, Karimundackal G, Laskar SG and Pramesh
- CS. Multimodality management of esophageal cancer. Indian J Surg 2014; 76: 494-503.
- [3] Du C, Fang M, Li Y, Li L and Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102: 33-42.
- Ilson DH. Esophageal cancer chemotherapy: recent advances. Gastrointest Cancer Res 2008; 2: 85-92.
- [5] Sun H, Nikolovska-Coleska Z, Lu J, Qin S, Yang CY, Gao W, Meagher J, Stuckey J and Wang S. Design, synthesis, and evaluation of a potent, cell-permeable, conformationally constrained second mitochondria derived activator of caspase (Smac) mimetic. J Med Chem 2006; 49: 7916-7920.
- [6] Sun H, Nikolovska-Coleska Z, Yang CY, Xu L, Liu M, Tomita Y, Pan H, Yoshioka Y, Krajewski K, Roller PP and Wang S. Structure-based design of potent, conformationally constrained Smac mimetics. J Am Chem Soc 2004; 126: 16686-16687.
- Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002; 9: 459-470.
- [8] Korga A, Korobowicz E and Dudka J. Role of mitochondrial protein Smac/Diablo in regulation of apoptotic pathways. Pol Merkur Lekarski 2006; 20: 573-576.
- [9] Qin S, Yang C, Li S, Xu C, Zhao Y and Ren H. Smac: Its role in apoptosis induction and use in lung cancer diagnosis and treatment. Cancer Lett 2012; 318: 9-13.
- [10] Qin S, Yang C, Wang X, Xu C, Li S, Zhang B and Ren H. Overexpression of Smac promotes cisplatin-induced apoptosis by activating caspase-3 and caspase-9 in lung cancer A549 cells. Cancer Biother Radiopharm 2013, 28: 177-182.
- [11] Xu Y, Zhou L, Huang J, Liu F, Yu J, Zhan Q, Zhang L and Zhao X. Role of Smac in determining the chemotherapeutic response of esophageal squamous cell carcinoma. Clin Cancer Res 2011; 17: 541254-22.
- [12] Endo K, Kohnoe S, Watanabe A, Tashiro H, Sakata H, Morita M, Kakeji Y and Maehara Y. Clinical significance of Smac/DIABLO expres-

sion in colorectal cancer. Oncol Rep 2009; 21: 351-355.

- [13] Arellano-Llamas A, Garcia FJ, Perez D, Cantu D, Espinosa M, Dela Garza JG, Maldonado V and Melendez-Zajgla J. High Smac/DIABLO expression is associated with early local recurrence of cervical cancer. BMC Cancer 2006; 6: 256.
- [14] Pluta A, Wrzesien-Kus A, Cebula-Obrzut B, Wolska A, Szmigielska-Kaplon A, Czemerska M, Pluta P, Robak T, Smolewski P and Wierzbowska A. Influence of high expression of Smac/DI-ABLO protein on the clinical outcome in acute myeloid leukemia patients. Leuk Res 2010; 34: 1308-1313.
- [15] Peng Cl, Hao Y, Zhao Y, Sun Q, Zhao X and Cong B. Effect of Smac and Taxol on non-small-cell lung cancer. Acta Biochim Biophys Sin (Shanghai) 2014; 46: 387-393.