Role of Smac in Determining the Chemotherapeutic Response of Esophageal Squamous Cell Carcinoma

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Smac, a mitochondrial apoptogenic protein, mediates apoptotic responses to various anticancer agents. Small-molecule mimetics of Smac have shown promising results in recent preclinical studies. Here we assessed the expression of Smac in tissues from patients with esophageal squamous cell carcinoma who had distinctly chemotherapeutic responses; we investigated the molecular mechanism by which Smac mediates chemosensitivity in ESCC cells. The results showed that Smac was significantly down-regulated in esophageal cancer, and differentially expressed in chemosensitive and chemoresistant tumors. Mechanistic studies revealed that Smac plays a pivotal role via the mitochondrial apoptotic pathway in modulating chemosensitivity of ESCC cells, and Smac mimetics sensitize cells to a chemotherapeutic response. Our data suggested that down-regulation of Smac may contribute to chemoresistance of esophageal cancer cells. Combinations of Smac mimetics and chemotherapeutic agents may have therapeutic benefits for the treatment of esophageal cancer.
Abstract

Purpose: Second mitochondria-derived activator of caspase (Smac) regulates chemotherapy-induced apoptosis. Smac mimetics have been tested in clinical trials as chemosensitizers. We determined the role of Smac in modulating the chemosensitivity of esophageal squamous cell carcinoma (ESCC).

Experimental Design: Smac expression was evaluated in tissues from ESCC patients with differential chemotherapeutic responses. The effects of Smac knockdown and Smac mimetics on the chemosensitivity of ESCC cells and the molecular mechanisms by which Smac and Smac mimetics modulate chemosensitivity were determined. The therapeutic responses of ESCC cells with different Smac statuses were compared using xenograft models.

Results: We found that Smac was significantly down-regulated in most ESCC samples (36.8%, 25/68, $P = 0.001$), and Smac expression differed significantly ($P < 0.05$) between chemosensitive and chemoresistant tumors. The associations of tested factors and their responses were examined using logistic regression analysis. In ESCC cells treated with cisplatin, a common chemotherapeutic drug, Smac and cytochrome c were released from mitochondria, and caspase-3 and caspase-9 were activated. Knockdown of Smac abrogated cisplatin-induced apoptosis, mitochondrial dysfunction, cytochrome c release, and caspase activation. Smac deficiency also reduced the effect of cisplatin on long-term cell viability, and led to cisplatin resistance in xenograft tumors in vivo. LBW242, a small-molecule Smac mimetic, enhanced cisplatin-induced apoptosis and caspase activation, and restored cisplatin...
sensitivity in Smac-deficient cells.

**Conclusion:** Our data suggested that down-regulation of Smac may be a chemoresistance mechanism in ESCC. Combinations of Smac mimetics with chemotherapeutic agents may have therapeutic benefits for the treatment of esophageal cancer.
Introduction

Esophageal cancer is the sixth most common cause of cancer-related death worldwide (1). Esophageal squamous cell carcinoma (ESCC), one of the major histopathological subtypes of esophageal cancer, is the fourth most prevalent malignancy in China (2, 3). Most ESCC patients are diagnosed with advanced diseases that responds poorly to chemotherapy. Concurrent chemo/radiotherapy is currently the standard of care in the nonsurgical management of advanced esophageal cancer (4). Cisplatin (cis-diaminedichloroplatinum (II)) combined with 5-fluorouracil (5-FU) is a classical regimen that is used to treat ESCC clinically. The anticancer effects of cisplatin are mediated via apoptosis induced by DNA adducts, which activate several proapoptotic signal transduction pathways (5).

Apoptosis is essential for normal development and plays an important role in the responsiveness to chemotherapy and radiotherapy. Apoptosis is triggered by two major intracellular signaling cascades the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway) (6). Both apoptotic pathways result in caspase activation and cleavage of specific cellular substrates, eventually leading to cell death. Apoptosis is tightly controlled by complex regulatory networks, and de-regulation of apoptosis contributes to pathological disorders such as cancer and therapeutic resistance to tumors (7).

The intrinsic apoptotic pathway is mediated by mitochondria and activated in response to intracellular stresses including oncogene activation and DNA damage. During the intrinsic apoptotic process, several apoptogenic proteins are released from
mitochondria into the cytosol to trigger downstream apoptotic events. Smac (also called DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein binding protein with low pI), a 23-kDa protein produced as a dimer within the intermembrane space of the mitochondria; Smac is released from mitochondria in response to anticancer drug stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins (IAPs) (8-11). Recent work suggests that Smac-mediated apoptosis is important for the apoptotic responses induced by several anticancer agents, including certain chemopreventive agents (10-14).

Accumulating evidence shows that Smac is a possible target for anticancer therapy. The combination of Smac activation with classical anticancer drugs seems to be a rational therapeutic approach (15, 16). Overexpression of Smac and chemically synthesized Smac mimetics can enhance apoptosis induced by chemotherapy and radiotherapy in several types of tumor cells (12, 13, 17-21). Smac expression has been detected in a variety of normal and tumor tissues, but the expression varies among different tumor types (22-26). However, little is known about the expression and role of Smac in esophageal cancer. We hypothesized that Smac expression is altered in ESCC and differential Smac expression contributes to differential chemotherapy response. To better understand the role of Smac in the chemotherapy of esophageal cancer, we assessed Smac expression with tissue microarrays (TMAs) of ESCC with immunohistochemical staining. We also investigated the molecular mechanisms by which Smac mediates cisplatin-induced apoptosis in esophageal cancer cells.
Materials and methods

Patients and tissue specimens. Surgical tissue specimens were collected from ESCC patients with informed consent and approval from the Institutional Review Board of the Cancer Institute and Hospital of Chinese Academy of Medical Sciences (CAMS), Beijing, China. For analysis of Smac expression, two TMAs with 86 ESCC cases were used. The TMAs included formalin-fixed and paraffin-embedded tumors and adjacent normal tissues from each patient. The first TMA had 56 ESCC cases, with 28 collected by the Cancer Institute and Hospital of Chinese Academy of Medical Science, and these latter 28 samples were from the high ESCC-incidence area in Yangquan, Shanxi province, China. All patients were diagnosed with ESCC by two senior pathologists and had not received chemotherapy and radiotherapy before surgery. The second TMA consisted of 30 ESCC cases, and was purchased from Outdo Biotech (Shanghai Outdo Biotech Co., China).

For 31 response cases of chemotherapy (26 male and 5 female, median 56 ranging from 38 to 73 years), included chemosensitive with complete response (CR)/partial response (PR) and chemoresistant with stable disease (SD)/progressive disease (PD), were obtained from in Cancer Hospital, CAMS between January, 1999 and December, 2007. All patients were diagnosed as ESCC histopathologically and subjected to surgical esophagectomy, followed by cisplatin-based chemotherapy for postoperative recurrence. Cisplatin was given by continuous intravenous administration at a dose of 60-100 mg/m² for 4 to 5 days, and in combination of 5-FU, Paclitaxel or Gemcitabine. The effect of chemotherapeutic response was evaluated clinically according to World
Health Organization (WHO) criteria. The diagnostic examinations consisted of esophagography, computed tomography, chest x-ray, abdominal ultrasonography and bone scan when necessary to detect recurrence or metastasis. All patient clinical parameters were summarized in supplementary Table 1 and Table 2.

**Immunohistochemical staining.** For IHC staining, the TMAs were incubated with an antibody against Smac or control IgG (1 μg/ml). After washing with PBS, TMAs were incubated with a biotin-labeled secondary antibody. Signals were visualized using an ultrasensitive streptavidin-peroxidase system (Maxim Biotech, Fuzhou, China). The intensity of Smac staining and the percentage of Smac-positive cells were scored as described (27). The results were scored independently by two pathologists based on the intensity of Smac staining and the extent of Smac expression. Intensity of Smac staining was scored as 0 (negative), 1 (weak), or 2 (strong). The extent of expression was scored based on the percentage of positive cells: 0 (negative), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The overall score of each case was a sum of the intensity of staining and extent of expression scores. Overall scores that are equal or higher than 4 were defined as positive staining, while those below 4 as negative staining.

**Chemotherapeutic drugs and chemicals.** The anticancer drugs used in this study, including cisplatin (QILU Pharmaceutical Co., China), paclitaxel (Bristol-Myers Squibb Co., USA), and 5-FU (Shanghai Xu Dong Hai Pu Medicine Co., China), were...
diluted in dimethylsulfoxide (Sigma., St. Louis, Mo, USA). The Smac mimetic NVP-LBW242-NX-5 (LBW242) was a gift from Novartis Pharmaceuticals Co. and was dissolved in DMSO to form 10 mmol/L stock solutions. 4’,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma dissolved in DMSO (2.5 mg/ml stock solution), and stored at –20°C. Propidium iodide (PI) was from BD Biosciences (San Jose, CA).

**Cell culture, drug treatment, and short hairpin RNA (shRNA) transfection.** The esophageal cancer cell lines EC0156 and KYSE510 (28) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (PAA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2. For drug treatment, cells were plated in 12-well plates at 20–30% confluence before treatment. For shRNA transfection, EC0156 cells were seeded in a 6-well plate at a density of 2 × 10^5 cells per well at least 20 h prior to transfection. The pSUPER-Smac-RNAi vector, which directs synthesis of siRNA targeting nucleotides 204–224 of the Smac coding sequence (TAGTAGTGAAGCATTGATGAG) (14) was transfected into EC0156 cells with Lipofectamine (Invitrogen, USA). After transfection, cells were placed in 96-well plates and selected with μg/ml puromycin (Life Technologies, Inc., Eggenstein, Germany). Smac expression in individual clones was analyzed by Western blotting. Stable clones with significant down-regulation of Smac were isolated.
Analysis of apoptosis. After treatment, both attached and floating cells were harvested at different times points, and washed with PBS. The fraction of apoptotic cells was determined by nuclear staining and two-color analysis with annexin V/PI. Nuclear morphology was assessed with DAPI staining. Briefly, cells were fixed with solution of 3.7% formaldehyde, 0.5% NP-40, and 10 μg/ml DAPI, and analyzed by fluorescence microscopy. Apoptotic cells with condensed chromatin and fragmented nuclei were counted from three different fields for per sample. All experiments were performed in triplicate. For annexin V/PI staining, the treated cells were stained using an annexin V/PI assay kit (Beijing Baosai Biological Technology Co., Ltd, China) and quantified. To detect changes in the mitochondrial membrane potential change, cells were harvested after treatment for 24 h, stained with Mito Tracker Red CMXRos (Invitrogen, USA) for 15 min at room temperature, and analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson).

Colony formation assay. After drug treatment, cells were trypsinized and seeded in 10-cm dishes (10^5 cells per dish) and cultured in DMEM supplemented with 10% FBS without anticancer drugs. After 14–16 days, cells were fixed in 3.7% formaldehyde and stained with 0.25% crystal violet (AMRESCO, USA) in PBS for 30 min. Clones were washed with water and counted. All experiments were performed in triplicate.

Western blot analysis. Cells were collected by centrifugation, washed with ice–cold PBS, and lysed in 30 mM Tris–HCl (pH 7.5) containing 1% Triton X–100, 0.15 M
NaCl, and cocktails (Roche, Mannheim, Germany) for 30 min. Lysates were homogenized through a 22–gauge needle and centrifuged at 10,000 × g for 10 min at 4°C. Proteins were separated on 12% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). Equal protein loading was confirmed with Coomassie Blue staining. Membranes were blocked with 5% fat-free milk in PBS containing 0.05% Tween-20, and incubated with dilutions of antibodies against caspase-3 (mouse IgG2a; BD Biosciences, Heidelberg, Germany), caspase-9 (mouse monoclonal, Oncogene, San Diego, CA), cytochrome c (mouse monoclonal antibody 7H8.2C12; BD Biosciences), AIF (rabbit polyclonal; Santa Cruz Biotechnology), or Smac (rabbit polyclonal) at 4°C overnight. Signals were detected with secondary antibodies conjugated to horseradish peroxidase, and visualized with the Renaissance Plus reagent (Santa Cruz).

**In vivo xenograft assay.** Briefly, 5 × 10^6 parental and Smac knockdown EC0156 (Smac-KD-G10) cells suspended in 50 μl PBS were injected subcutaneously into both flanks of female nude mice (n = 5). When the tumor size reached approximately 60 mm³, the mice were intraperitoneally injected with 3 mg/kg of cisplatin. The tumor size was measured every other day after cisplatin treatment, and tumor volume was calculated using the formula 0.5 × a × b², in which, “a” is the longest dimension of the tumor and “b” is perpendicular to a.

**Statistical analysis.** Statistical analysis was carried out with SPSS software (standard
version 14.0; SPSS Inc. Chicago, IL, USA). Comparisons of quantitative data were analyzed with the Student’s $t$-test between two groups in the mouse xenograft assay. Categorical data were analyzed with the Chi-square test to compare the expression of Smac between tumor and normal tissues on the TMAs. Difference were considered significant for $P < 0.05$. The logistic regression analysis was used to assess the statistical significance of the association between the expression of Smac and chemotherapy response.
Results

Smac expression is decreased in ESCC and correlates with the chemotherapeutic response.

To assess the levels of Smac in ESCC and adjacent normal tissues, we analyzed Smac expression in TMAs of ESCC using IHC staining. Of 86 ESCC cases analyzed, 68 were informative. Smac was expressed in 36.8% (25/68) of ESCC samples and in 64.7% (44/68) of matched adjacent normal tissues. Smac downregulation in ESCC tumor tissues compared to normal tissues was statistically significant (chi-square test, \( P = 0.001 \)). Smac was detected in the cytoplasm of ESCC and normal cells, with a definitive pattern in most tissues (Figure 1). Smac expression levels did not correlate with tumor histological grade or gender. All of the available clinical parameters were summarized in supplementary Table 3.

To investigate whether Smac expression correlated with the chemotherapeutic response, we analyzed 31 ESCC cases who had undergone cisplatin-based chemotherapy (Figure 1 and Table 1). Smac expression was significantly associated with the chemotherapeutic response. Positive Smac staining was significantly more intense (\( P = 0.019 \)) in the CR/PR groups compared to the SD/PD groups (Table 1). However, the presence of Smac in these ESCC tissues did not obviously correlate with other clinical and pathologic characteristics, including the patients’ age, gender, and tumor histopathological features (Table 1).
Cisplatin induces apoptosis in esophageal cancer cells.

To determine whether Smac-mediated apoptosis is related to chemosensitivity of esophageal cancer cells, EC0156 cells were treated with cisplatin at different concentrations followed apoptosis analysis using several methods. Nuclear condensation and fragmentation, typical features of apoptotic cells, were detected 24 h after cisplatin treatment (Figure 2A). The levels of apoptosis were dose- and time-dependent, reaching about 60% and 80% after treatment with 10 μM cisplatin for 24 and 48 h, respectively (Figure 2B). Cisplatin-induced apoptosis in EC0156 cells as seen with annexin V/PI staining (Figure 2C), decreased mitochondrial membrane potential collapse as detected with Mito Tracker Red staining (Figure 2D), and decreased long-term cell viability as seen with colony formation assay (Figure 2E). We also found that cisplatin induced apoptosis in KYSE510 cells (data not shown).

To examine how cisplatin induced apoptosis in EC0156 and KYSE510 cells, we analyzed the release of mitochondrial apoptogenic proteins. Cytosolic fractions were isolated and analyzed for the expression of Smac and cytochrome c with Western blotting. Smac and cytochrome c significantly accumulated in the cytosolic fractions of both cell lines following cisplatin treatment for 24 h (Figure 2F). As a fractionation control, the mitochondrial inner membrane protein cytochrome oxidase subunit IV (COX IV) was not detected in the cytosolic extracts. Because Smac can promote caspase activation through competitive binding with IAPs subsequent to its release from mitochondria (9, 29), we analyzed caspase activation in cells treated with...
cisplatin. The active form of caspase-3, (a 17-kDa fragment required for execution of apoptosis), was found in EC0156 and KYSE510 cells undergoing cisplatin-induced apoptosis (Figure 2F). Thus, cisplatin induced apoptosis via the intrinsic mitochondrial pathway in ESCC cells.

**Smac knockdown decreases cisplatin-induced apoptosis and causes cisplatin resistance.**

To investigate the role of Smac in cisplatin-induced apoptosis, shRNA was used to knock down Smac expression in EC0156 cells. After shRNA transfection and isolation of stable drug-resistant clones, Smac knockdown was analyzed with Western blotting. Smac was absent in Smac-KD-G10 and Smac-KD-E1 cells, compared to the parental EC0156 cells and a control sister clone (Figure 3 A and B). After treatment of the parental and Smac-KD-G10 cells with cisplatin for 24 h, apoptosis was analyzed with nuclear staining and annexin V/ PI staining. Smac-KD cells were more resistant to cisplatin-induced apoptosis than the parental cells (Figure 3C). Apoptosis induced by different concentrations of cisplatin was significantly decreased in the absence of Smac (Figure 3D). Furthermore, apoptosis induced by paclitaxel and 5-FU was also inhibited by Smac knock-down (Figure 3C). Annexin V/ PI staining showed that the Smac-KD cells had reduced numbers of annexin V– positive cells (15.04%) compared with parental cells (69.6%) (Figure 3E). Analysis of long-term cell viability with colony formation assay showed that Smac-KD cells treated with cisplatin for 6 h had increased long-term survival compared with the parental EC0156 cells. Thus, Smac
deficiency significantly decreased cisplatin-induced apoptosis and led to cisplatin resistance.

**Smac deficiency decreases cisplatin-induced caspase activation, cytochrome c release, and mitochondrial membrane potential change.**

To determine the mechanism by which Smac mediates cisplatin-induced apoptosis, parental and Smac-KD EC0156 cells were treated with 10 μM cisplatin. Caspase-9 activation was markedly reduced in Smac-KD cells compared to parental cells following cisplatin treatment for 12 h (Figure 4A). Activation of caspase-3 barely changed in Smac-KD cells, in contrast to the parental cells (Figure 4A). Therefore, Smac contributed to cisplatin-induced caspase-9 activation. Because the release of cytochrome c is thought to be the major mechanism of caspase-9 activation (6, 30), we compared cytochrome c release in the parental and Smac-KD EC0156 cells treated with cisplatin. Importantly, cytochrome c release was attenuated in Smac-KD cells compared to parental cells in response to cisplatin (Figure 4A), consistent with a previous study (14). The mitochondrial membrane potential change was also blocked in Smac-KD cells compared with parental cells (Figure 4B). Thus, inhibition of cisplatin-induced apoptosis by Smac deficiency is related to inhibition of cytochrome c release.

**Smac deficiency leads to cisplatin resistance in vivo.**

To determine whether Smac deficiency causes cisplatin resistance in vivo, an equal
number of parental and Smac-KD EC0156 cells were injected subcutaneously into both flanks of nude mice. One week after inoculation, tumor-bearing mice were treated with 3 mg/kg cisplatin on days 1, 3, and 5. The antitumor efficacy was measured by monitoring the tumor volume after treatment. Cisplatin treatment only caused a slight, nonsignificant reduction in the size of the Smac-KD tumors, relative to the tumors treated with vehicle alone ($P = 0.592$; Figure 4C); in contrast, the size of the parental EC0156 tumors was significantly decreased ($P = 0.001$; Figure 4C). Thus, Smac deficiency can cause cisplatin resistance in vivo.

**Smac mimetics restore cisplatin-induced apoptosis in Smac-KD cells.**

Smac binds to IAPs through its N-terminal four residues (AVPI) to relieve the inhibition of caspase-9 and caspase-3 (31, 32). Smac mimetics can induce degradation of cellular IAP1 and sensitize cancer cells to apoptosis induced by anticancer agents (33-35). Therefore, we explored whether Smac mimetics could restore cisplatin-induced apoptosis in Smac-KD cells by analyzing LBW242, a Smac mimic of AVPI (12, 19). LBW242 greatly sensitized Smac-KD cells to cisplatin-induced apoptosis, but had a smaller effect on the parental EC0156 cells (Figure 5A, B), as confirmed by annexin V/PI staining (Figure 5C). LBW242 also enhanced caspase-9 activation and cytochrome c release in response to cisplatin treatment (Figure 5D). Thus, activation of the mitochondrial apoptotic pathway by Smac mimetics can potentially overcome cisplatin resistance in Smac-deficient cells.

Taken together, our results demonstrated that Smac expression was down-regulated
in ESCC, and this down-regulation was significantly associated with poor response to chemotherapy. Smac plays a critical role in execution of cisplatin-induced apoptosis in ESCC cells by regulating cytochrome c release and caspase activation.

Discussion

Approximately half of patients diagnosed with esophageal cancer present with overt metastatic disease, and advanced metastatic disease is treated with chemotherapy. ESCC patients often receive combination chemotherapy involving conventional chemotherapeutics such as cisplatin, 5-FU, etoposide, and paclitaxel (4). Despite the use of the cisplatin/5-FU combination, there is no convincing evidence for significant improvement in the overall survival rate. Previous studies showed an association between Smac expression and tumor progression, poor prognosis, and altered responses to diverse stimuli in many human cancers (25, 36-40). However, the role of Smac in ESCC remains unclear. Thus, we studied Smac in ESCC patients, including those who had undergone chemotherapy. We observed Smac was frequently down-regulated in ESCC, with only 36.8% (25/68) of ESCC tissues expressing Smac, compared to 64.7% (44/68) of matched adjacent normal tissues. Thus, Smac downregulation may contribute to esophageal carcinogenesis. Reduced Smac expression has also been described in other types of cancer (11, 38, 40-42). Importantly, our data revealed a significant association between Smac expression and chemotherapeutic response among the 31 ESCC patients who had undergone chemotherapy. Smac staining was observed more frequently in the CR+PR group than
that in the SD+PD group ($P=0.019$). The lower expression of Smac and correlation between Smac expression and chemotherapeutic response in ESCC patients suggest that loss of Smac function may contribute to the intrinsic resistance of esophageal cancer to chemotherapy and radiotherapy.

Our functional analysis indicates that Smac plays a critical role in chemotherapy-induced apoptosis in ESCC cells. Smac, and cytochrome c, were released into cytosol in response to chemotherapy. Smac knockdown significantly suppressed cisplatin-induced apoptosis, mitochondrial membrane potential collapse, caspase activation, and cytochrome c release, leading to cisplatin resistance in vitro and in vivo. This critical role of Smac in chemotherapy-induced apoptosis is also supported by previous studies on Smac using other agents and different cell types (13, 43-46). Apoptosis is a major cytotoxic mechanism of chemotherapeutic agents, and defective apoptosis contributes to therapeutic resistance. Therefore, Smac downregulation in ESCC may compromise the apoptotic effects of chemotherapeutic agents, resulting in therapeutic resistance. Further understanding of the precise etiology of chemoresistance in ESCC requires additional insight into how Smac and other apoptosis regulators mediate apoptosis induced by anticancer drugs.

The effects of Smac release on mitochondrial membrane potential and cytochrome c release suggest that Smac may participate in a feedback loop to promote further mitochondrial dysfunction for full execution of apoptosis. Release of both Smac and cytochrome c results in caspase activation. Although Smac neutralizes IAPs to relieve inhibition on caspases, cytochrome c mediates the assembly of the apoptosome. The
relationship between Smac and cytochrome c is controversial. Several studies have shown that both proteins are coordinately released upon apoptotic stimulation (47, 48), yet other studies indicate that cytochrome c and Smac release can occur separately through independent mechanisms (49). We found that Smac deficiency in ESCC cells significantly attenuated cytosolic accumulation of cytochrome c and blocked activation of caspase-9 in cisplatin-induced apoptosis. The reduction of cytochrome c release in Smac-deficient cells may be due to a requirement for Smac in directly promoting cytochrome c release or a lack of an influence of Smac on mitochondrial integrity, which is important for cytochrome c release. In contrast to our observations, a previous study showed that Smac release induced by high concentration of anticancer drugs was suppressed in cytochrome c-deficient mouse embryonic fibroblast cells (50). Apoptosis induced by multiple apoptotic stimuli is in Smac-deficient mice, suggesting a redundant role for Smac and compensation for the absence of Smac function by other molecules during apoptosis (51). Therefore, the requirement for Smac in apoptosis appears to be dependent on specific stimuli and cell types.

The mitochondrial apoptotic pathway is generally thought to involve translocation of cytochrome c from mitochondria into cytosol, which results in activation of caspase 9 and caspase 3. However, cytochrome c-independent activation of caspases has also been described. For example, Greil and his colleagues have shown that activation of caspase 9 by resveratrol does not require cytochrome c release (52). Hishita and his colleagues reported that caspase 3 could be activated by lysosomal
enzymes in cytochrome c-independent apoptosis induced by etoposide in P39 cells (53). It has been shown that procaspase-9 is a substrate of caspase 12, and that ER stress induced a specific cascade response involving caspasess 12, 9, and 3 in a cytochrome c-independent manner (54). In our study, we could not detect cytochrome c release in EC0156 cells treated with cisplatin for 12 h, despite the clear effects of cisplatin on the mitochondrial membrane potential and caspase activation. This observation may be explained by cytochrome c-independent early activation of caspase 9 or caspase 3 in EC0156 cells treated with cisplatin for 12 h. At a later time point (24 h), the release of cytochrome c may enhance the activation of caspase 9 and caspase 3, and further amplify apoptotic signals.

Structural and biochemical studies have shown that the proapoptotic function of Smac is mediated through its N-terminal AVPI motif, which binds to one or more IAP repeat domain in IAPs and antagonizes IAP-mediated inhibition of caspase activation (18, 19). Smac overexpression and agents that mimic the AVPI motif can sensitize cancer cells to several chemotherapeutic agents (55-59). We found that LBW242, a 7-mer small-molecule Smac mimetic (19), enhanced apoptosis induced by chemotherapeutic agents in ESCC cells, and restored cisplatin sensitivity in Smac-deficient cells. These activities of LBW242 were accompanied by increased caspase-9 activation and cytochrome c release, suggesting that the effects of Smac on apoptosis occur through a feedback loop and enhanced cytochrome c release. Recent studies have shown that small-molecule mimetics of Smac can kill cancer cells via a different mechanism by inducing autoubiquitination and subsequent degradation of
IAPs (34, 35, 60). The precise mechanisms by which Smac mimetics sensitize chemotherapy-induced apoptosis remain to be further defined.

In conclusion, our results demonstrate down-regulation of Smac, association of Smac expression with chemotherapeutic response, and a critical role of Smac in cisplatin-induced apoptosis in ESCC. These results suggest that combinations of conventional anticancer drugs with Smac mimetics may be beneficial for the treatment of esophageal cancer, especially Smac-deficient tumors. Further studies of Smac and other apoptosis regulators may help develop novel therapeutic strategies for esophageal cancer.
Conflicts of interest

None declared.

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Figure legends

Figure 1. Immunohistochemical analysis of Smac in ESCC and normal epithelial tissues on TMAs. (A), (B) Down-regulation of Smac in ESCC. Expression of Smac was analyzed using TMAs containing 68 cases of ESCC and adjacent normal epithelial tissues, with duplicate cores for each case. The TMAs were stained with Smac polyclonal antibodies. The majority of normal esophageal epithelia showed positive staining in the cytoplasm. Lower cytoplasmic Smac expression was detected in the corresponding esophageal cancer tissues. (C)-(F) Smac expression in ESCC with differential chemotherapeutic response. The esophageal cancers with CR (C) and PR (D) responses to cisplatin chemotherapy expressed higher levels, whereas those with SD (E) and PD (F) responses expressed lower levels of Smac. Magnification, 200×; inserts, 400×.
Figure 2. Apoptosis induced by cisplatin in esophageal cancer cells. (A) Apoptosis induced by cisplatin was quantified following nuclear staining. EC0156 cells were treated with 10 μM cisplatin for 24 h. Nuclei were stained with DAPI. The appearance of apoptotic cells was significant 24 h after exposure to cisplatin. (B) Dose- and time-dependent apoptosis induced by cisplatin. EC0156 cells were treated with the indicated concentration of cisplatin for 24 or 48 h. (C) Apoptosis was quantified following annexin V/PI staining. EC0156 cells were treated with cisplatin (10 μM and 20μM) for 24 h, stained with annexinV/PI, and analyzed with flow cytometry. Four subpopulations are indicated, including necrotic cells (R1), late apoptotic cells (R2), viable cells (R3), and early apoptotic cells (R4). (D) EC0156 cells were treated with 10 μM cisplatin for 24 h, stained with Mito Tracker Red CMXRos, and analyzed by flow cytometry. (E) EC0156 cells were treated with 10 μM cisplatin for 6 h. Long-term cell viability was assessed with the colony formation assay. (F) Cisplatin-induced caspase activation and Smac and cytochrome c release in ESCC cells. EC0156 and KYSE510 cells were treated with 10 μM cisplatin for 12 or 24 h. Upper, mitochondrial and cytosolic fractions were isolated from the treated cells and analyzed for the indicated proteins with Western blotting. Alpha-tublulin was used as a control for fraction and loading. Lower, total cell lysates were probed for the cleavage of caspase-3 with Western blotting. Arrow indicates the 17 kDa caspase-3 cleavage fragment.
Figure 3. Inhibition of cisplatin-induced apoptosis in Smac-KD EC0156 cells. (A) RNAi knockdown of Smac in EC0156 cells. EC0156 cells were transfected with a Smac shRNA construct. Smac knocked down puromycin-resistant clones were identified with Western blotting, Beta-actin was the loading control. (B) Quantification of Smac expression in (A) using the Quantity One program. (C) Inhibition of apoptosis in Smac-KD cells. Parental cells, Smac-KD cells (G10 and E1 clones), and a control puromycin-resistant EC0156 clone with Smac expression (D7) were treated with 10 μM cisplatin for 24 h. Apoptosis was quantified by fluorescence microscopy after nuclear staining with DAPI. (D) Parental and Smac-DK-G10 EC0156 cells were treated with the indicated concentrations of cisplatin for 24 h to induce apoptosis. Apoptosis was analyzed by nuclear staining as in (C). Results are the averages of three independent measurements, with a minimum of 200 cells counted for each measurement. (E) Parental and Smac-DK-G10 EC0156 cells were treated with 10 μM cisplatin for 24 h. stained with annexin V/PI, and analyzed by flow cytometry. Four subpopulations are indicated, including necrotic cells (R1), late apoptotic cells (R2), viable cells (R3), and early apoptotic cells (R4). (F) Long-term cell viability in parental and Smac-KD cells. After parental and Smac-KD-G10 EC0156 cells were treated with 10 μM cisplatin for 6 h, long-term cell viability was assessed by the colony formation assay. Representative pictures are shown.
Figure 4. Smac mediates cisplatin-induced apoptosis through the mitochondrial pathway; cisplatin sensitivity of ESCC tumors in vivo. (A) Cisplatin-induced caspase activation and cytochrome c release in Smac-KD cells. Parental and Smac-KD-G10 EC0156 cells were treated with 10 μM cisplatin for 12 or 24 h. Smac and cytochrome c release were analyzed by Western blottting of lysates of the treated cells. Alpha-tubulin was used as a loading and fractionation control. Mitochondrial fractions were isolated from the treated cells and analyzed for cytochrom c and Cox IV with Western blottting. Total cell lysates were assayed for the cleavage of caspase-9 and caspase-3 with Western blottting. The cleavage fragments were indicated by arrows. Beta-actin was the loading control. (B) Mitochondrial membrane potential in Smac-KD cells. Parental and Smac-KD-G10 cells were treated with 10 μM cisplatin for 24 h, harvested and stained with Mito Tracker Red CMXRos, and analyzed by flow cytometry. (C) Suppression of the antitumor effects of cisplatin in Smac knockdown in mice. Parental and Smac-KD-G10 EC0156 cells suspended in 0.2 ml PBS were injected into nude mice (female, 6 -weeks old) to establish xenograft tumors. After the tumor volume reached 100 to 200 mm^3, mice were treated with 3 mg/kg cisplatin in 0.1 ml PBS, or with vehicle alone, by i.p. injection once every other day for a week. Tumor volume was measured every 2–3 days after treatment. Cisplatin treatment significantly decreased the growth of the parental tumors (\(P < 0.001\)), but had no significant effects (\(P > 0.05\)) on Smac-KD tumors. Values are means ± SD (\(n = 5\) in each group). **\(P < 0.001\); # \(P > 0.05\).
**Figure 5.** The Smac mimetic LBW242 restores cisplatin-induced apoptosis in Smac-KD cells. (A), (B) Apoptosis induced by cisplatin combined with LBW242. Parental EC0156 and Smac-KD-G10 cells were treated with 10 μM cisplatin with or without 10 μM LBW242 for 24 h. Apoptosis was quantified by fluorescence microscopy after nuclear staining with DAPI. (C) Following treatment as in (B), cells were stained with annexin V/PI, and apoptosis was quantified by flow cytometry. Four subpopulations are indicated, including necrotic cells (R1), late apoptotic cells (R2), viable cells (R3), and early apoptotic cells (R4). (D) Caspase-9 activation in Smac-KD cells treated with cisplatin and LBW242. After treatment as in (B), cells were lysed and assayed for caspase-9 with Western blotting. Beta-actin was the loading control. (E) Cytochrome c release in Smac-KD cells treated with cisplatin and LBW242. Following treatment as in (B), cytosolic fractions were isolated from the treated cells and analyzed for cytochrome c with Western blotting. Alpha-tubulin was the loading and fractionation control.
References


A) Western blot analysis showing expression levels of Smac and Actin in different cell lines.

B) Graph displaying the rate of Smac expression in EC0156, Smac-KD G10, Smac-KD E1, and Smac-KD D7 cell lines.

C) Bar graph showing the percentage of apoptotic cells in Taxel, 5-FU, and Cisplatin-treated cells, comparing EC0156 and Smac-KD G10.

D) Line graph illustrating the increase in apoptotic cells with varying concentrations of Cisplatin, comparing EC0156 and Smac-KD G10.

E) Flow cytometry analysis of Annexin V-FITC staining in untreated and Cisplatin (10 μM)-treated cells, comparing EC0156 and Smac-KD G10.

F) Petri dishes showing cell growth inhibition in Cisplatin (10 μM)-treated EC0156 and Smac-KD G10 cells.
Table 1. Correlation of chemotherapeutic response and clinicopathological features with Smac expression.

<table>
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<th>Variable</th>
<th>N=31</th>
<th>Expression of Smac</th>
<th>P value</th>
<th>OR (95%CI)</th>
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<td></td>
<td>Total</td>
<td>Positive (%)</td>
<td>Negative (%)</td>
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</tr>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>26</td>
<td>11 (42.3)</td>
<td>15 (57.7)</td>
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<tr>
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<td>5</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>0.364 2.9 (0.28-30.0)</td>
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<tr>
<td>≤ 60</td>
<td>18</td>
<td>9 (50)</td>
<td>9 (50)</td>
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<tr>
<td>&gt; 60</td>
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<td>3 (23.1)</td>
<td>10 (76.9)</td>
<td>0.137 3.3 (0.68-16.3)</td>
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<td>Histological grade</td>
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<td>13 (72.2)</td>
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<tr>
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<td>7 (53.8)</td>
<td>6 (46.2)</td>
<td>0.062 0.23 (0.049-1.078)</td>
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<td>7 (70)</td>
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<tr>
<td>SD+PD</td>
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<td>5 (23.8)</td>
<td>16 (76.2)</td>
<td>0.019 7.47 (1.385-40.245)</td>
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</table>

Values are expressed as n (%). Statistically significant at P<0.05.

*OR = odds ratio; CI = confidence interval; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.
Role of Smac in determining the chemotherapeutic response of esophageal squamous cell carcinoma

Yang Xu, Lanping Zhou, Jing Huang, et al.

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