Clinical significance of Smac/DIABLO expression in colorectal cancer

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Abstract. Second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) is released by mitochondria in response to apoptotic stimuli and is thought to regulate apoptosis by antagonizing inhibitors of apoptosis proteins, which play an important role in sensitization of cancer cells to various therapeutic regimens. The expression of Smac/DIABLO has been demonstrated in various cancer cells, though little is known about its clinical significance with respect to colorectal cancer. The current study was designed to evaluate the relationship between prognosis and Smac/DIABLO expression by clinicopathological analysis of patients with colorectal cancer. Smac/DIABLO expression was evaluated using immunohistochemical staining in 121 consecutive patients with colorectal cancer and the relationship between Smac/DIABLO expression and clinicopathological factors was analyzed. Smac/DIABLO-positive expression was detected in 80 of the 121 patients (66%). The incidence of lymph node and distant metastasis in Smac/DIABLO-negative cancer was significantly higher than that in Smac/DIABLO-positive cancer (P=0.0004 and P=0.003, respectively). While univariate analysis showed that survival in patients with Smac/DIABLO-negative expression was significantly poorer than in Smac/DIABLO-positive cases (P<0.0001), Smac/DIABLO-negative expression was a prognostic indicator independent of Dukes' staging and lymph node metastasis by multivariate analysis. This study proposes that the decrease of Smac/DIABLO expression is an independent factor determining the poorer prognosis of patients with colorectal cancer.

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Introduction

Mitochondria are vital for cellular biogenetics, playing a central role in determination of the point-of-no-return of the apoptotic process, such that they are considered to be therapeutic targets for cancer chemotherapy in recent times (1). In response to various apoptotic stimuli, mitochondria have been shown to release death proteins (2,3), such as cytochrome c (cyto-c) (4,5), AIF (6), Endo G (7) and HtrA2/Omi (8). Once released into the cytosol, these mitochondrial proteins trigger both caspase-dependent and -independent apoptosis. Conversely, inhibitors of apoptosis proteins (IAPs) block the activity of caspases that promote apoptotic cell death (9,10) with overexpression of IAPs being related to chemoresistance in various tumor cells (11-13).

Second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO: SMAC hereafter) (14,15) is a death protein released from mitochondria that is known to antagonize the function of IAPs (16).

Of the novel mitochondrial factors (4-8,14,15), SMAC is recognized as a potent therapeutic target since it is a direct antagonist of anti-apoptotic IAPs (17,18).

The level of expression of SMAC and its role in treatment sensitization has been studied in relation to several types of cancers. SMAC levels were seen to correlate well with survival in lung cancer (19), while overexpression of it increased chemosensitivity in gastric cancer cells (20), hepatocellular carcinoma (21) and osteosarcoma (22). Low levels of SMAC resulted in early resistance to chemotherapy in thyroid cancer (23) and its expression was downregulated in renal cell carcinoma, predicting a poor prognosis (24). In the case of colon cancer, increased apoptosis correlated with an increased release of SMAC in HCT116 and SW480 colon cancer cells, a couple of previous studies suggesting that decreased levels of SMAC may be important in chemoradiation-resistance in advanced colon cancer (25-27). The actual role of SMAC in colorectal cancer, however, has been ill defined.

The current study aimed to elucidate the biological significance of SMAC expression and investigated its potential as a potent prognostic and biological marker in colorectal cancer.

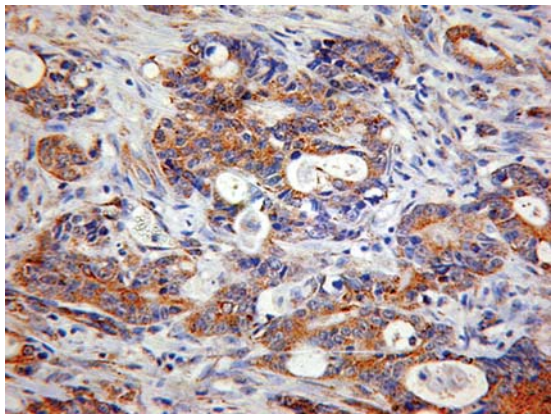
Patients and methods

Patients. A total of 121 consecutive patients with colorectal cancer who had undergone surgery at Department of General Surgery, Fukuoka Dental College Hospital and its affiliated hospitals between January 1994 and May 2000 were included in this study. The study population consisted of 63 men and 58 women and their ages ranged from 31 to 84 years with a mean of 65. Specimens were collected from the above patients, embedded in paraffin and stained to provide representative sections of each specimen. The patients were followed up and only those who died of colorectal cancer were regarded as having died of tumor-related causes. The follow-up interval after surgery ranged from 54 days to 16 years and 11 months, with a mean of 6 years and 4 months. Clinicopathological results were assessed according to the general rules for clinical and pathological studies on cancers of the colon, rectum and anus outlined by the Japanese Research Society for Cancer of the Colon and Rectum (28).

Immunohistochemical staining. Immunohistochemical staining was performed on the paraffin-embedded specimens using the peroxidase-labeled streptavidin-biotin technique with the Histone SAB-PO kit (Nichirei). Two consecutive sections of 4 μm thickness were prepared from each sample. One section was stained with hematoxylin and eosin (H&E). The other was subjected to specific immunostaining with anti-SMAC antibody (IMG-248, Imgenex, San Diego, CA, USA), a polyclonal antibody that is reactive with human SMAC (29). Tissue sections were deparaffinized in xylene, rehydrated with a series of graded ethanols and placed in phosphate-buffered saline (PBS) for 10 min. After cooking the slides in a pressure cooker in citrate buffer solution (pH 6.0) for 6 min, to retrieve the antigen, endogenous peroxidase activity was blocked for 30 min with methanol containing 0.3% hydrogen peroxidase. Sections were then incubated with 10% non-immunized rabbit serum for 10 min to block nonspecific binding of the immunoreagents and incubated overnight at 4˚C with rabbit anti-human polyclonal SMAC antibody at a 1:50 dilution. The sections were subsequently incubated with a second stage biotinylated antibody for 20 min, followed by incubation with horseradish peroxidase-labeled streptavidin for 20 min at room temperature. After washing in PBS, localization of SMAC was visualized with diaminobenzidine tetrahydrochloride.

All the stained sections were analyzed by two observers. In addition, the results were assessed by a pathologist, who had not been given any clinical information about the sections. If the percentage of positive-staining cancer cells accounted for <30% of the total number of cancer cells, the respective patients from whom the specimens had been collected were classified as SMAC-negative, while if the number of stained cells exceeded 30%, patients were classified as SMAC-positive regardless of the intensity of staining.

a



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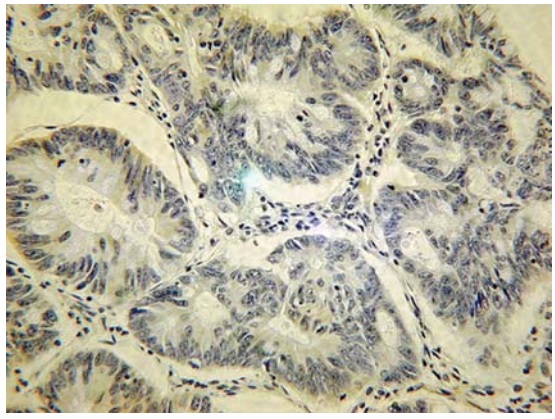

Figure 1. (a) SMAC-positive immunostaining of moderately differentiated adenocarcinoma of the colon showing cytoplasmic expression of carcinoma cells (x200). (b) SMAC-negative immunostaining in moderately differentiated adenocarcinoma of the colon (x200).

Statistical analysis. The 121 patients were divided into two groups, depending on whether Smac/DIABLO expression was positive or negative and compared for clinicopathological characteristics using the Chi-square and Student's t-tests. Cumulative survival rates were evaluated by the Kaplan-Meier method and the survival curves were tested by the Mantel-Cox method. Multivariate survival analysis was done according to Cox's proportional hazards model in a forward stepwise manner. A p-value <0.05 was considered statistically significant.

Results

SMAC was predominantly localized in the cytoplasm of cancer cells (Fig. 1a and b). The relationship between SMAC expression and the clinicopathological features of the patients are shown in Table I. Based on the results of immunohistochemical staining, 80 of 121 patients (66%) were positive for SMAC expression.

Clinicopathological features between SMAC-positive and -negative groups were compared. The incidence of lymph node metastasis was 15/80 (18.75%) in the SMAC-positive group, which was significantly lower than the 19/41 (46.3%) in the SMAC-negative group (P=0.003). Distant metastases,

Table I. SMAC expression and clinicopathological characteristics.

Variables	SMAC-positive (n=80) (%)	SMAC-negative (n=41) (%)	P-value
Gender			
Male	45 (56.25)	18 (43.9)	N.S.
Female	35 (43.75)	23 (56.1)	
Age (years)	64.5±12.2	67.3±11.8	N.S.
Tumor size (mm)	49.1±20.4	51.2±25.3	N.S.
Location of tumors			
Cecum and ascending colon	14 (17.5)	7 (17.1)	N.S.
Transverse colon	8 (10.0)	3 (7.3)	
Descending colon	4 (5.0)	1 (2.4)	
Sigmoid colon	26 (32.5)	19 (46.4)	
Rectum	28 (35.0)	11 (26.8)	
Pathological type			
Well	45 (56.25)	24 (58.5)	N.S.
Moderately	23 (28.75)	14 (34.2)	
Poorly	5 (6.25)	2 (4.9)	
Mucinous	7 (8.75)	1 (2.4)	
Tumor depth			
Mucosa	3 (3.75)	1 (2.4)	N.S.
Submucosa	6 (7.5)	2 (4.9)	
Muscularis	13 (16.25)	4 (9.8)	
Subserosa	25 (31.25)	8 (19.5)	
Serosa	31 (38.75)	20 (48.8)	
Invading surrounding organs	2 (2.5)	6 (14.6)	
Lymph node metastasis			
Positive	15 (18.75)	19 (46.3)	0.003
Negative	65 (81.25)	22 (53.7)	
Lymphatic permeation			
Positive	18 (22.5)	10 (24.4)	N.S.
Negative	62 (77.5)	31 (75.6)	
Venous invasion			
Positive	20 (25.0)	13 (31.7)	N.S.
Negative	60 (75.0)	28 (68.3)	
Distant metastasis			
Positive	1 (1.25)	9 (22.0)	0.0004
Negative	79 (98.75)	32 (78.0)	
Dukes' classification			
A	19 (23.75)	6 (14.6)	0.0001
B	45 (56.25)	13 (31.7)	
C	15 (18.75)	13 (31.7)	
D	1 (1.25)	9 (22.0)	

N.S., not significant; well, well differentiated adenocarcinoma; modereately, moderately differentiated adenocarcinoma; poorly, poorly differentiated adenocarcinoma; mucinous, mucinous adenocarcinoma and SMAC, Smac/DIABLO.

including liver and lung metastasis, were recognized in a total of 10 patients, 9 of whom belonged to the SMAC-negative group. Tumor staging according to Dukes' criteria was performed. The proportion of patients classified as stage C and D in the SMAC-positive group was significantly lower than that in the negative group (16/80, 20.0% and 22/41, 53.7%, respectively) while the proportion of patients classified as stage A and B was significantly higher in the positive as compared to the negative group (64/80, 80.0% and 19/41, 46.3%; P=0.0001). No significant differences were noted between the two groups with respect to tumor size, tumor location, histological differentiation and lymphatic and

Table II. Factors independently associated with prognosis.

Variables	Standard error	Odds ratio	CI 95%	P-value
Lymphatic invasion	0.510	0.577	0.274-2.025	0.564
Venous invasion	0.509	0.699	0.258-1.899	0.484
SMAC expression	0.569	4.460	4.150-38.659	<0.001
Dukes' stage	0.829	3.320	0.013-0.324	<0.001
Lymph node metastasis	0.758	2.516	1.524-29.777	0.012

CI, confidence interval and SMAC, Smac/DIABLO.

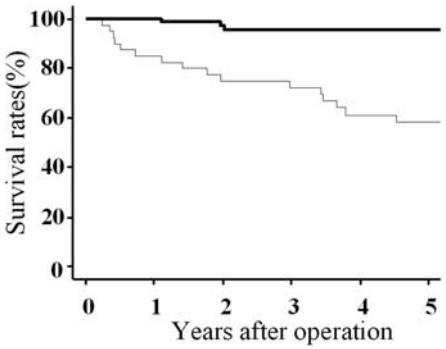

Figure 2. Survival curve of colorectal cancer in SMAC exhibiting and non-exhibiting patients. The prognosis of patients was significantly worse in SMAC-negative patients (thin line) as compared to patients who were SMAC-positive (thick line) (P<0.0001).

venous invasion. Thus, negative expression of SMAC correlated with aggressiveness of the cancer.

One-, 3- and 5-year survival rates of patients in the SMAC-positive group were 98.7, 96.0 and 96.0%, respectively, which were significantly higher than survival rates of patients in the SMAC-negative group (82.5, 72.2 and 58.1%, respectively; p<0.0001) (Fig. 2). Multivariate analysis demonstrated that SMAC expression, Dukes' staging and lymph node metastasis were independent prognostic factors in patients with colorectal cancer (Table II).

Discussion

The data presented here indicate that SMAC expression significantly correlated with lymph node metastasis, distant metastasis and Dukes' staging. Furthermore, it was an independent prognostic factor taking rank with lymph node metastasis and Dukes' staging, the expression of SMAC being inversely related to the aggressiveness of the cancer. A strong explanation for the above results is the close relationship between SMAC expression and its ability to regulate apoptosis.

IAPs such as XIAP are strongly expressed in various cancers including colon cancer (30) and are associated with poor prognosis and resistance to apoptosis (11,31). XIAP is the most potent and best characterized member of the mammalian IAP family (32), being a predominant SMAC binding protein. SMAC binds to XIAP, displaces XIAP from caspase-9, promotes cleavage of effector caspases and induces apoptosis (33,34). Several previous studies have shown that increased apoptosis correlates with an increased release of SMAC in colon cancer cells (25-27).

Hence, it can be safely hypothesized that decreased or lack of SMAC expression is related to increased aggressiveness of a cancer and that SMAC modulates malignant behavior through interaction with IAPs.

Another explanation in support of SMAC's important role in cancer may be its ability to regulate the metastatic potential of a cancer. Huerta *et al* reported that SMAC expression was diminished in metastatic colon cancer cells, making it a potential target for chemosensitization in the treatment of advanced colon cancer (35).

In the current study, 9 out of 10 cases with distant metastasis had negative expression of SMAC. Distant or lymph node metastases are significant prognostic factors in colon cancer (36), reflecting the ability of SMAC to affect the progression of cancer through promoting metastasis.

Many studies relating to SMAC and colon cancer have suggested that stimulation of apoptosis causes concomitant release of three important mitochondrial pro-apoptotic factors with different mechanisms of action: a) release of cytochrome c, which mediates caspase-3 activation with formation of the apoptosome; b) release of AIF which mediates DNA fragmentation through caspase-independent pathway; and c) release of SMAC, which neutralizes the activity of IAPs. The relative role and ratios of these factors in the process of apoptosis are important, though as yet unclear (4-6,37). SMAC is therefore believed to regulate carcinogenesis in colon cancer by virtue of its ability to neutralize IAPs.

In conclusion, immunohistochemical detection of SMAC expression is a potent prognostic marker in colorectal cancer, although its biological function is still not completely clear. Elucidation of such a function may contribute to our search for a new strategy against cancer progression and metastasis. Recent studies with SMAC peptides (SMAC-mimetics) have shown that it could be useful in the development of more effective chemopreventive strategies and agents for various types of cancer cells including colon cancer (17,18,38-40). This may lead to human trials with specific therapies targeting SMAC in advanced or inoperable colorectal cancers refractory to conventional chemoradiotherapies.

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