FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 48, No. 4, 2010
pp. 678-681

Prognostic significance of smac/DIABLO in endometrioid endometrial cancer

Bozena Dobrzycka[1], Slawomir J. Terlikowski[1], Piotr S. Bernaczyk[2], Magdalena Garbowicz[3], Jacek Niklinski[3], Lech Chyczewski[2], Marek Kulikowski[4]

[1]Department of Obstetrics, Gynecology and Obstetrics / Gynecological Care
[2]Department of Pathomorphology
[3]Department of Clinical Molecular Biology
[4]Department of Perinatology, Medical University of Bialystok, Poland

Abstract: Apoptosis may occur via a death receptor-dependent or independent (mitochondrial) pathway. The mitochondrial pathway is regulated by small molecules, such as smac/Diablo, which activates caspase cascades. This study examined smac/DIABLO expression in 76 patients with endometrioid endometrial cancers. Presence of smac/DIABLO was quantified by Western blot analysis using nonfixed fresh frozen tissues. Its appearance was found in 55 (72%) of examined tumors. Smac/DIABLO expression significantly correlated with tumor grade ($p<0.001$). Patients with positive smac/DIABLO tumors had a longer disease-specific survival when compared with those with negative tumors in the 10-year follow-up ($p=0.043$). The study demonstrated that negative smac/DIABLO expression was a poor prognostic sign.

Keywords: endometrioid endometrial cancer, smac/DIABLO, prognosis

Introduction

In multicellular organisms, the total number of cells is a balance between the cell-generating effects of mitosis and cell death that is induced through apoptosis. A disruption of this delicate balance can lead to the development of cancer [1].

The first descriptions of apoptosis were made over 150 years ago, although the implications for tumor development were not appreciated until the 1970s. Natural cell death is a critical part of development of multicellular organisms, and also counter-balances the cell generating effects of mitosis [2]. Mitochondria play a key role in the apoptotic process; their damage, which involves permeabilization of the outer mitochondrial membrane, activates a series of events that lead to cell death. Of the two proposed signaling pathways of apoptosis, i.e. the "extrinsic" and the "intrinsic" pathway, the latter is assumed to initiate in mitochondria. Its activation involves release of cytochrome c and other pro-apoptotic factors from the mitochondrial intermembrane space [3].

Smac (second mitochondria derived activator of caspase) and its murine ortholog DIABLO (Direct IAP binding with Low pI) are mitochondrial proteins encoded by nuclear DNA which are released into the cytosol in response to apoptotic stimuli that disrupt the integrity of mitochondria [4]. Smac/DIABLO participates in the two main apoptotic pathways, the intrinsic or mitochondrial pathway [5] and the extrinsic or death receptor pathway [6]. After apoptotic stimuli, released smac acts as a dimer in the cytosol, activating caspases by means of sequestering and neutralizing members of the inhibitor of apoptosis proteins family (IAPs) [7].

Although the expression of smac/DIABLO has been reported in various cancers, little is known about its clinical significance in endometrial cancer. The current study was designed to evaluate the relationship between prognosis and smac/DIABLO expression by clinicopathological analysis of patients with endometrioid endometrial cancer.

Materials and methods

Patients and clinical samples. A total of 76 patients with endometrioid endometrial cancers (aged 46-67; median value –

Correspondence: B. Dobrzycka, Department of Obstetrics, Gynecology and Obstetrics/Gynecological Care, Medical University of Bialystok, Warszawska 15, 15 062 Bialystok, Poland; tel.: (+4885) 74 88 869, fax.: (+4885) 74 88 860, e mail: bdobrzycka@gmail.com

Folia Histochem Cytobiol. 2010:48(4): 678 (678-681)
Doi: 10.2478/v10042-010-0091-2

Table 1. Western blot analysis of smac/DIABLO expression compared with stage and grade of examined endometrioid endometrial cancer.

		No. of cases	smac/DIABLO no. (%)
Stage	I	43	31 (72)
	II	24	17 (71)
	III	9	7 (78)
p value			p=0.288
Grade	1	41	36 (88)
	2	21	14 (65)
	3	14	5 (36)
p value			p<0.001

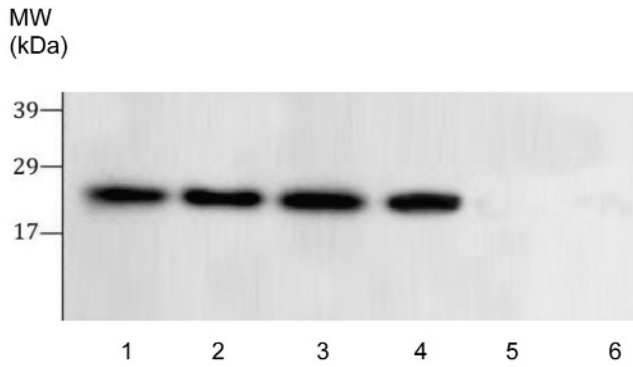

Fig. 1. Western blot analysis of smac/DIABLO after 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis. MW (kDa) – size marker, samples 1, 2, 3 and 4- tumors with smac/DIABLO expression; samples 5 and 6 – tumors with absence of protein.

59.4 years) treated at the Department of Gynecology and Septic Obstetrics Medical University of Bialystok and the Department of Gynecology District Hospital in Bialystok between 1999 and 2003 were included in this study. None of the patients had received chemotherapy, hormonal therapy or radiation therapy prior to surgery. All patients had primary cancers and were receiving first treatment. Cases selected in the present study showed the same stage, both clinically and surgically. All tumors were staged according to the FIGO criteria.

Clinicopathological information was obtained from medical charts. Histopathological examination was performed according to the WHO classification. Representative samples of hysterectomy specimens were stained with H+E for light microscopic study and evaluated to confirm a tumor stage and histological type.

Immunohistochemistry. Tissue sample was frozen in liquid nitrogen and maintained at 70°C for Western blot analysis. The presence of smac/DIABLO protein was estimated by immunoblotting with an anti-smac/DIABLO mouse monoclonal antibody (BD Biosciences, USA, No. 612244). For Western blot analysis, tissue samples were suspended in 0.05 M Tris HCl buffer (pH 7.6) in the ratio 1:3 (wt/vol). The homogenates were prepared with a knife homogenizer (25000 rpm for 45 seconds at 4°C) and sonicated (20 kHz, 4 × 15 seconds at 4°C). After centrifugation (10000 × g for 15 minutes at 4°C), supernatants were stored at -70°C until measurements were performed. Supernatants were equilibrated with a loading buffer (10% sodium dodecylsulfate [SDS] in Tris HCl [pH 8.0], containing 50% glycerol, 0.1 mM 2-β-mercaptoethanol and 0.1% bromophenol blue) at 50 μg/mL. The mixture was then denatured for 5 minutes at 100°C and samples containing 20 μg of protein were subjected to electrophoresis. The following molecular mass standards were used: 39, 29 and 17 kDa (Bio Rad Laboratories, USA). The gels were allowed to equilibrate in 25 mM Tris, 0.2 M L glycine, 20% (vol/vol) methanol for 5 minutes and proteins were transferred to 0.2 μm pore diameter nitrocellulose membranes at 100 mA for 1 hour. The membranes were then incubated with one of the following primary antibodies at dilutions 1:500 in 5% dried defatted milk in TBS T (20 mM Tris HCl buffer, pH 7.4; 150 mM NaCl; 0.05% (vol/vol) Tween 20) for 1 hour. Species specific secondary antibodies were then added at 1:7500 dilutions. Incubation was continued for 30 minutes with slow shaking. Then, nitrocellulose membranes were washed with TBS T (5 times for 5 minutes) and treated with Sigma Fast BCIP/NBT reagent (Sigma Aldrich, Germany). The labeled membranes were photographed, scanned, and optical density was analyzed using imagining QuantityOne software (Bio Rad Laboratories, USA).

Ethical issues. Patients were informed and gave their consent for the study. The protocol was previously approved by the Bioethical Committee of the Medical University of Bialystok. Follow-up data were completed until January 2010.

Statistical analysis. Statistical analysis was performed using Statistica software version 9.0PL (StatSoft, Inc., StatSoft Polska Sp. z o.o., Poland). A chi-square test was used to evaluate the relationship between categorical variables. Fisher's exact test was used to determine significance between the two groups. A p-value of <0.05 was considered as statistically significant. In addition, survival time was calculated from the date of surgery to the date of death and survival analysis was performed using the Kaplan-Meier method.

Results

Among 76 patients with endometrioid endometrial carcinomas, 43 had tumors classified as stage I, 24 patients had tumors classified as stage II, and 9 patients were classified as stage III. The samples were grouped by histological grade: 41 were classified as grade 1, 21 were grade 2 and 14 were grade 3 (Table 1). All patients were followed either until death; at a median follow-up of 60 (range 1-126) months.

Using anti-human smac/DIABLO antibody, presence of protein was observed in 55 of 76 (72%) endometrioid endometrial carcinomas (Fig 1). When the smac/DIABLO expression in the tumor samples were analyzed, there were significant differences between histological grade (p<0.001). We found no significant correlation between protein expression and clinical tumor stage (p=0.288) (Table 1).

One-, 5- and 10-year survival rates of patients in the smac/DIABLO-positive group were 97.8, 95.5 and 95.5%, respectively, what is significantly higher than survival rates of patients in the smac/DIABLO-negative group (93.7, 78.1 and 75%, respectively; p=0.043) (Fig. 2).

Folia Histochem Cytobiol. 2010:48(4): 679 (678-681)
Doi: 10.2478/v10042-010-0091-2

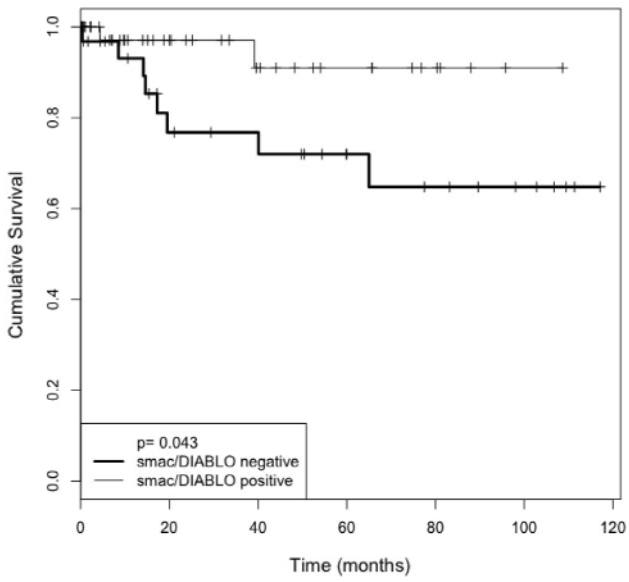

Fig. 2. Kaplan-Meier survival analysis concerning smac/DIABLO expression and cumulative survival in endometrioid endometrial cancer patients. The prognosis of patients was significantly worse in smac/DIABLO-negative patients (thick line) as compared to patients who were smac/DIABLO-positive (thin line) (p=0.043).

Discussion

Smac/DIABLO is released from mitochondria into the cytosol during apoptosis, promoting caspase activation by neutralizing the inhibition of inhibitor of apoptosis proteins (IAPs) on caspases. As demonstrated by Western blot analyses, the expression of smac/DIABLO is widespread, suggesting its role in the regulation of apoptosis in a variety of cell types [8, 9]. It has also been reported that some carcinoma cells overexpress both pro- and antiapoptotic proteins and that a marginal increase in expression of one proapoptotic compound can tip the overall balance in favor of cell death [10].

The level of expression of smac/DIABLO and its role in prognosis has been studied in relation to several types of cancers. The protein expressions were seen to correlate well with poor prognosis in lung cancer [11], colorectal cancer [12-14], hepatocellular carcinoma [15] and sarcomas from various origins [16]. Low smac/DIABLO expression in renal cell carcinoma predicted a poor prognosis [17-19]. In bladder cancer it may be associated with resistance to chemotherapy [17]. Thus, alteration of apoptosis is essential for cancer development and cancer cell death by radiation and chemotherapy is largely dependent upon apoptosis [16]. These data partially contradict those of others that smac/DIABLO expression does not correlate with stage, grade and prognosis [20,21].

In the present report, we have shown for the first time that smac/DIABLO protein expression correlates with tumor grade and survival of patients with endometrioid endometrial cancer. Since smac/DIABLO expression could be used as a prognostic parameter in patients with endometrioid endometrial cancer, the accurate prediction of prognosis may help in selecting patients for more intensive treatment. We suggest that detection of smac/DIABLO is a potent prognostic marker in endometrioid endometrial cancer, although its biological function is still not completely clear.

References

[1] Cotter TG. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer.* 2009;9(7):501-507.
[2] Pettigrew CA, Cotter TG. Deregulation of cell death (apoptosis): implications for tumor development. *Discov Med.* 2009;8(41):61-63.
[3] Caroppi P, Sinibaldi F, Fiorucci L, *et al*. Apoptosis and human diseases: mitochondrion damage and lethal role of released cytochrome C as proapoptotic protein. *Curr Med Chem.* 2009;16(31):4058-4065.
[4] Martinez-Velazquez M, Melendez-Zajgla J, Maldonado V. Apoptosis induced by cAMP requires Smac/DIABLO transcriptional upregulation. *Cell Signal*. 2007;19(6):1212-1220.
[5] Verhagen AM, Ekert PG, Pakusch M, *et al*. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell.* 2000;102(1):43-53.
[6] Srinivasula SM, Datta P, Fan XJ, *et al*. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem.* 2000;275(46):36152-36157.
[7] Wilkinson JC, Wilkinson AS, Scott FL, *et al*. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. *J Biol Chem.* 2004;279(49):51082-51090.
[8] Lu S, Xu W, Fan Z, *et al*. Overexpression of Smac/DIABLO in Hep-2 cell line: possible role in potentiating the sensitivity of chemotherapeutic drugs. *Tumori*. 2010;96(2):310-315.
[9] Hansen TM, Smith DJ, Nagley P. Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome c. *Cell Death Differ*. 2006; 13(7): 1181-1190.
[10] Yang L, Cao Z, Yan H, *et al*. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. *Cancer Res.* 2003;63(20):6815-6824.
[11] Sekimura A, Konishi A, Mizuno K, *et al*. Expression of Smac/DIABLO is a novel prognostic marker in lung cancer. *Oncol Rep.* 2004;11(4):797-802.
[12] De Oliveira Lima F, De Oliveira Costa H, Barrezueta LF, *et al*. Immunoexpression of inhibitors of apoptosis proteins and their antagonist SMAC/DIABLO in colorectal carcinoma: correlation with apoptotic index, cellular proliferation and prognosis. *Oncol Rep.* 2009;22(2):295-303.
[13] Endo K, Kohnoe S, Watanabe A, *et al*. Clinical significance of Smac/DIABLO expression in colorectal cancer. *Oncol Rep*. 2009;21(2):351-355.
[14] Anguiano-Hernandez YM, Chartier A, Huerta S. Smac/DIABLO and colon cancer. *Anticancer Agents Med Chem*. 2007;7(4):467-473.
[15] Bao ST, Gui SQ, Lin MS. Relationship between expression of Smac and Survivin and apoptosis of primary hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int.* 2006;5(4):580-583.

Folia Histochem Cytobiol. 2010:48(4): 680 (678-681)
Doi: 10.2478/v10042-010-0091-2

[16] Yoo NJ, Kim HS, Kim SY, *et al*. Immunohistochemical analysis of Smac/DIABLO expression in human carcinomas and sarcomas. *APMIS*. 2003;111(3):382-388.
[17] Mizutani Y, Katsuoka Y, Bonavida B. Prognostic significance of second mitochondria-derived activator of caspase (Smac/DIABLO) expression in bladder cancer and target for therapy. *Int J Oncol*. 2010;37(2):503-508.
[18] Mizutani Y, Nakanishi H, Li YN, *et al*. Overexpression of XIAP expression in renal cell carcinoma predicts a worse prognosis. *Int J Oncol.* 2007;30(4):919-925.
[19] Yan Y, Mahotka C, Heikaus S, *et al*. Disturbed balance of expression between XIAP and Smac/DIABLO during tumour progression in renal cell carcinomas. *Br J Cancer*. 2004;91(7):1349-1357.
[20] Shibata T, Mahotka C, Wethkamp N, *et al*. Disturbed expression of the apoptosis regulators XIAP, XAF1, and Smac/DIABLO in gastric adenocarcinomas. *Diagn Mol Pathol*. 2007;16(1):1-8.
[21] Arellano-Llamas A, Garcia FJ, Perez D, *et al*. High Smac/DIABLO expression is associated with early local recurrence of cervical cancer. *BMC Cancer.* 2006;6:256.

Submitted:18 August, 2010
Accepted after reviews: 1 October, 2010

Folia Histochem Cytobiol. 2010:48(4): 681 (678-681)
Doi: 10.2478/v10042-010-0091-2