doi:10.4149/neo_2011_05_430

Correlation of Smac/DIABLO protein expression with the clinico-pathological features of breast cancer patients

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Received February 9, 2011

Smac/DIABLO protein promotes caspase-dependent apoptosis by inhibition of inhibitor of apoptosis protein (IAP) family members. The role of Smac/DIABLO in breast cancer has not been yet established. Therefore, the aim of the study was to assess the expression of this protein in tumor cells from breast cancer patients. The expression of Smac/DIABLO was analyzed in 62 breast cancer patients by flow cytometry. The obtained results were compared with expression of this protein in benign breast tumor tissue, which served as the control (11 patients with fibroadenoma). Expression of caspase-3 proteins in breast cancer was also evaluated. Smac/DIABLO expression in breast cancer was correlated with clinical and pathological data. Although the expression of Smac/DIABLO protein was found in all examined samples of both the breast cancer and fibroadenoma patients, the median expression of Smac/Diablo in breast cancer was significantly lower than in the control (39.1% vs. 48.1%; p=0.0047). Smac/DIABLO expression correlated with expression of caspase-3 (p=0.000008). In pT1 breast cancer patients, expression of Smac/DIABLO protein was higher than in those with pT2-3 (p=0.02). Diffuse cancer infiltration significantly correlated with lower expression of Smac/DIABLO protein (p=0.02). Moreover, there was a loose correlation between low expression of Smac/DIABLO protein and cancer embolus in minor blood and lymphatic vessels (p=0.08). Our results indicate that expression of Smac/DIABLO inversely correlates with the tumor stage, which may suggest that this protein may play an important role in the breast cancer development.

Key words: breast cancer, Smac/DIABLO protein, apoptosis, flow cytometry

Breast cancer is the most common malignancy in the female population throughout the world (1). Early diagnosis and multimodal therapy play a key role in the improvement of prognosis in these patients. The decision about type of treatment is based on the presence of prognostic and predictive factors such as age and menopausal status, stage of the disease, histological grade, estrogen and progesterone receptor status, human epidermal growth factor receptor 2 (HER2) as well as the involvement of lymphatic vessels and veins in the vicinity of the tumor (2). However, further understanding of the molecular pathways of breast cancer development may be crucial for the introduction of optimal treatment tailored to each patient, which may also include biologically-targeted therapy.

The suppression of apoptosis is considered an important mechanism leading to cancer formation and progression (3). The important apoptosis-regulating proteins are inhibitors of apoptosis protein (IAP) family members, which are responsible for the inhibition of the proteolytic activity of caspases 9 and 3, as well as their antagonists (4-8). Among the proteins inhibiting the IAP-dependent caspase-activation are Smac/DIABLO (second mitochondrial-derived activator of caspase/ Direct IAP Binding Protein with Low PI), HtrA2/OMI (high temperature requirement A) and XAF-1 (XIAP-associated factor 1) (Figure 1). One of the most frequently studied IAP antagonists is the Smac/DIABLO protein. Due to the pro-apoptotic role of Smac/DIABLO, it could be expected that its expression negatively correlates with cancer progression. Nevertheless, the level of Smac/DIABLO in tumor cells has been demonstrated by Yoo et al. to vary among different types of malignancies (9). In their study Smac/DIABLO was not detected in prostate, lung and soft tissue cancers, which may reflect impaired apoptosis of these tumor cells. In contrast, gastric, colorectal and ovarian cancer cells showed high mitochondrial expression of Smac/DIABLO. However, in those tumors this protein is

Table 1. Clinico-pathological features of breast cancer patients group (n=62).

Feature	Data
Age	
– Under 50 years	9 (14,5%)
– 50 years and more	53 (85,5%)
Tumor stage	
– pT1	32 (51%)
– pT2	27 (44%)
– pT3	3 (5%)
– pT4	0
Lymph node status	
– N negative (pN0)	30 (49%)
– N positive (pN1-2)	31 (51%)
Histology	
– Infiltrating ductal carcinoma	65 (90%)
– Infiltrating lobular carcinoma	6 (10%)
Histologic grade	
– G1	3 (5%)
– G2	27 (44%)
– G3	25 (40%)
– Unknown	7 (11%)
Diffuse cancer foci	
– Yes	4 (6%)
– No	58 (94%)
Lymphatic and/or vein infiltration	
– Yes	9 (14,5%)
– No	53 (85,5%)
ER	
– Negative	17 (27%)
– Positive	42 (68%)
– Unknown	3 (5%)
PR	
– Negative	21 (34%)
– Positive	38 (61%)
– Unknown	3 (5%)
HER2	
– Negative	39 (63%)
– Positive	13 (21%)
– Unknown	10 (16%)

not released from mitochondria to cytosol, probably due to inhibition of anti-apoptotic Bcl-2 protein (9,10). Expression of SMAC/Diablo protein in the breast cancer and its correlation with clinico-pathological features of disease has not been yet established.

In this study, we assessed the expression of Smac/DIABLO protein in native breast cancer tissue using flow cytometry methods. To our knowledge this is the first study describing application of this technique in Smac/DIABLO expression assessment in solid tumors. To verify whether expression of Smac/DIABLO in breast cancer is linked to apoptosis in these cells, expression of this protein was compared with caspase-3 activation. Additionally, the expression of Smac/DIABLO was assessed in correlation with known prognostic and predictive factors in breast cancer.

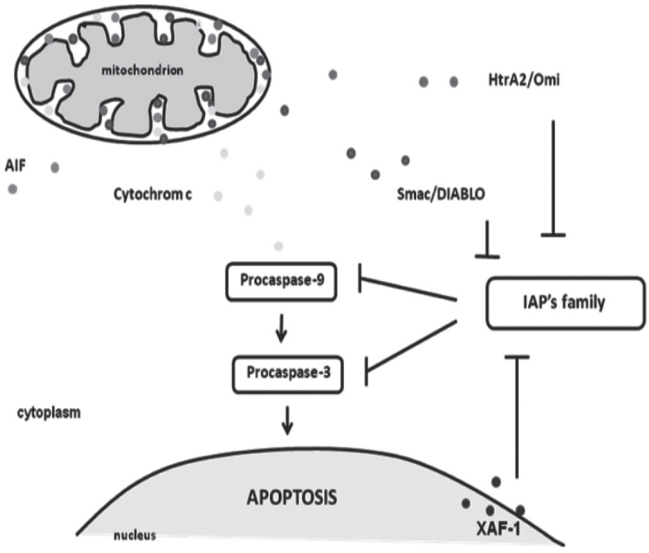

Figure 1. Smac/DIABLO apoptosis pathway.

Patients and methods

Patients. Sixty two women with primary breast cancer who had undergone surgery in the Department of Surgical Oncology, Medical University of Lodz between February 2007 and September 2009 were included to the study group. The median age was 59 years (range from 41 to 88 years). Pathological inclusion criteria encompassed infiltrating ductal carcinoma – not otherwise specified (NOS) and infiltrating lobular carcinoma. Patients with preoperative chemotherapy or hormonotherapy, patients with other pathological types of breast cancer and non-invasive breast cancer were excluded. The clinical and pathological features of the study group are summarized in Table 1.

The control group consisted of 11 female patients operated because of fibroadenoma of the breast. Their ages range from 24 to 53 years with a median of 30. Patients with a cancer history were excluded.

Methods

Sample processing. Breast tumor tissues were collected from patients during surgery. Native tumor specimens, approximately 0.5 -1 cm^3 of volume, were stored at -80° C . Single cells were isolated from defrosted samples and prepared for flow cytometry measurement according to the method described by Ehemann et al (11). Briefly, fragmented native tissues were re-suspended in 2.1% citric acid/0.5% Tween 20, and gently shaken at room temperature. Cell suspensions were fixed in 70% ethanol and phosphate buffered saline (PBS; Sigma Aldrich Chemie Gmbh, Steinheim, Germany).

Before flow cytometry analysis, cells were washed in PBS, centrifuged (5min, 1100rpm) and incubated in 0.01% saponin

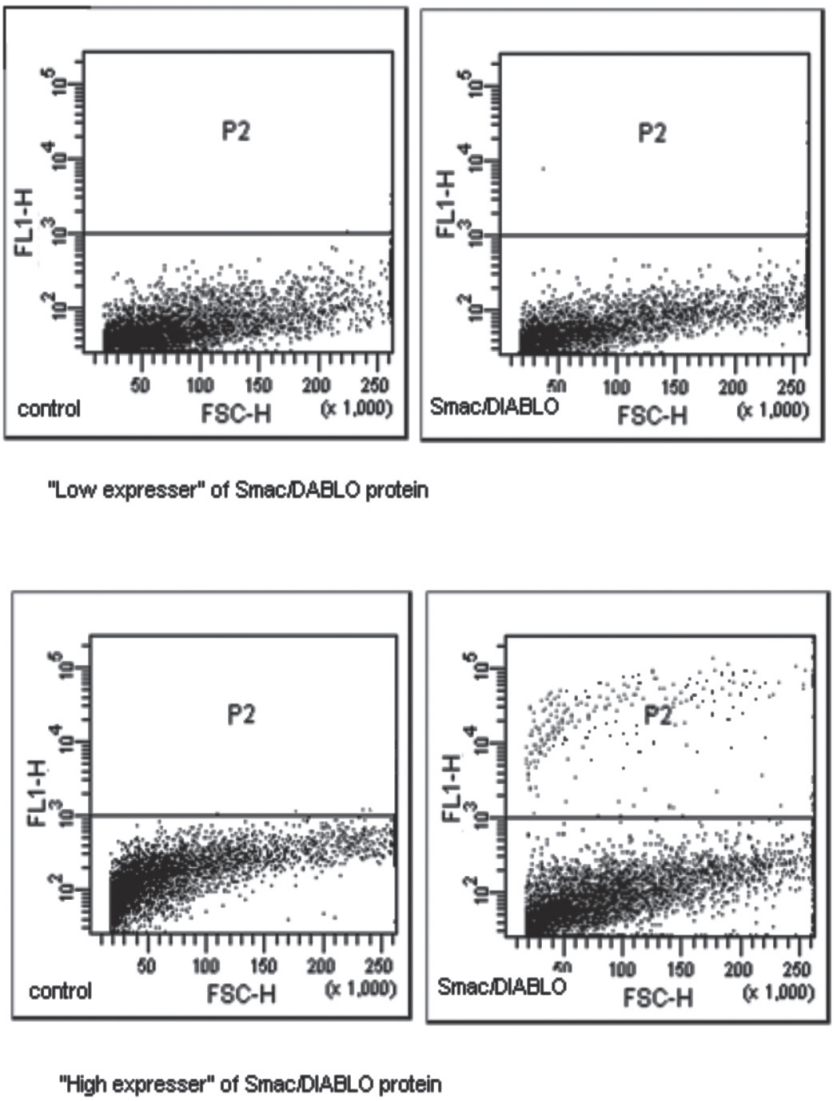

Figure 2. Expression of Smac/DIABLO protein in Flow Cytometry.

for 1 min. The cells were then washed in PBS and centrifuged again (5 min, 1100 rpm). Incubation with primary antibody at a dilution of 1: 100 (anti-Smac/DIABLO, polyclonal goat Ab, R&D System, Minneapolis, MN, USA; anti-cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) was performed at 4°C, overnight. On the following day, samples were washed in PBS, centrifuged (5min, 1100rpm) and incubated for 120min with secondary FITC-conjugated Abs at dilution 1:20. Afterwards, samples were washed in PBS, centrifuged (5min, 1100rpm), and then resuspended in 400ul PBS and subjected to flow cytometry analysis. Simultaneously, samples with isotype controls were prepared (Normal Goat IgG control, 1:100 dilution, R&D System, Minneapolis, MN, USA).

Flow cytometry analysis. All measurements were performed using flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA). An acquisition gate was established based on FSC (forward scatter) versus SSC (side scatter) distribution that included cells according to previous immunophenotype (cytokeratine 19). Cell fluorescence was measured using standard emission filters: FL1 (green, 515-545nm), FL2 (orange, 564-606 nm). For each analysis 10,000 events were acquired and analyzed using CellQuestPro software (Becton Dickinson, San Jose, CA, USA). The Smac/DIABLO-positive cells were identified after gating based on appropriate isotype controls. Expression of Smac/DIABLO was presented as a percentage of Smac/DIABLO-positive cells in the whole population of tumor cells. Similarly, expression of caspase-3 was established as a percentage of caspase-3 positive cells in the whole tumor cell population (Figure 1). All flow cytometry measurements were performed on 10,000 cells per sample. "High" and "low"

expression was established based on the median of Smac/DIABLO and cleaved caspase-3-positive cells estimated in the whole group of patients (Figure 2).

Statistical analysis. Statistical analysis was performed with Statistica 7.0 (Tulusa, OK, USA) software. Statistical comparison of "low-expressers" and "high-expressers" was performed using the Mann-Whitney *U* test. Correlations between variables were assessed by the Spearman rank correlation coefficient (r). Comparisons between examined parameters were considered significant when p <0.05.

Results

Expression of Smac/DIABLO protein. Smac/DIABLO protein expression was found in all breast cancer patients (62/62; 100%). Expression ranged from 1.2% to 76.7% with a median of 39.1%. Also, expression of the analyzed protein was present in all members of the control group (11/11;100%) : median expression 48.1%; range 42.1-68.7%. Expression of Smac/DIABLO protein in breast cancer was significantly lower than in the control samples (p=0.005).

Expression of Smac/DIABLO protein and caspase-3 protein in breast cancer samples. Expression of cleaved caspase-3 proteins was found in 61 out of a total 62 breast cancer patients (61/62; 98%). Median expression of cleaved caspase-3 in breast cancer patients was 5.8% and ranged from 0 to 42.3%. In all patients from the control group, cleaved caspase-3 protein expression was revealed with median expression 8.1% (range 6-19.5%). Expression of cleaved caspase-3 protein in breast cancer showed trend towards lower expression compared to the controls (p=0.07). In the study group expression of Smac/DIABLO protein significantly correlated with caspase-3 expression (p=0.000008; r=0.53).

Relationship between expression of Smac/DIABLO protein and clinico-pathological characteristics of the study group. Expression of Smac/DIABLO protein was summarized in Table 2. The protein levels were higher in the subgroup of cancer patients under 50 years old (p=0.05). In breast cancer pathologically assessed as pT1, expression of Smac/DIABLO protein was higher than in pT2-3 (p=0.02). A relationship was found between the presence of diffuse breast cancer infiltration in postoperative specimens and a lower expression of Smac/DIABLO protein (p=0.02). Also, a lower expression of Smac/DIABLO protein tended to correlate with the occurrence of the cancer embolus in minor blood and lymphatic vessels (p=0.08). Metastases in axillary lymph nodes did not correlate with expression of Smac/DIABLO protein in tumor cells (p=0.91) (Table 2).

Table 2. Relationship between Smac/DIABLO expression and known prognostic factors in breast cancer (*U* Manna-Whitneya test)

Feature	Smac/DIABLO expression Median (range)	*p*-value
Age (years)		
<50	43,9 (2,3-68,7)	p=0.05
>=50	40,4 (1,2-76,7)	
Tumor stage		
pT1	43,8 (1,2-76,7)	p=0.02
pT2 and pT3	29,9 (3,8-63)	
Lymph node status		
Negative	35,7 (2,3-76,7)	p=0.91
Positive	38,4 (3,8-63,8)	
Histological grade		
G1	42,2 (1,2-76,7)	p=0.73
G2 and G3	30,1 (3,8-63)	
Diffuse cancer foci		
Yes	12,6 (2,3-33,7)	p=0.02
No	39,8 (1,2-76,7)	
Lymphatic and/or vein infiltration		
Yes	26,35 (3,8-55,7)	p=0.08
No	39,8 (1,2-76,7)	
ER		
Negative	33,5 (3,8-63)	p=0.92
Positive	38,4 (1,2-76,7)	
PR		
Negative	40,4 (3,8-63)	p=0.41
Positive	30,8 (1,2-76,7)	
HER2		
Negative	40,4 (1,2-76,7)	p=0.15
Positive	33,3 (2,3-55,7)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human growth factor receptor 2.

Discussion

This is a first report describing the expression of Smac/DIABLO protein in breast cancer patients. The study showed expression of Smac/DIABLO in all examined samples obtained from patients with this tumor.

Previous studies indicated that expression of Smac/DIABLO varies among cancer types. In real-time RT-PCR assessment, Smac/DIABLO mRNA was expressed in all assessed (n=85) renal cell carcinoma (RCC) patients (12). In the Western blot analysis, 82% RCC samples expressed Smac/DIABLO protein (13). The expression of Smac/DIABLO was assessed immunohistochemically in 100 carcinomas and 50 sarcomas of various organs by Yoo et al.[9]. The highest levels, reaching 70% of evaluated tumors, were seen in gastric adenocarcinoma, colorectal adenocarcinoma and ovarian serous adenocarcinoma. A moderate rate of Smac/DIABLO expression (about 40%) was seen in hepatocellular carcinoma and lung cancer patients (9,14). The lowest expression, about 20%, was seen in prostate cancer patients [9]. With regard to hematological malignancies, expression of Smac/DIABLO was confirmed in almost all (96%) acute myeloid leukemia patients (15). Ren et al. found this protein in 63% of Hodgkin lymphoma patients and only in 47% of non-Hodgkin lymphoma patients (16). These diversities of Smac/DIABLO expression in cancers suggest that only in some malignancies the Smac/DIABLO protein may have an important role in impairment of the mechanisms of cell death.

Despite the fact that expression of Smac/DIABLO was present in all breast cancer samples, the median expression of this protein was reduced. We reported that median expression of Smac/DIABLO protein was lower in breast cancer tissues than in fibroadenoma (39.1% vs. 48.1%; p=0.005). This observation indicates that in benign tumors, the mechanisms of programmed cell death might work more efficiently.

Since Smac/DIABLO protein is involved in mechanisms leading to apoptosis, it might be expected that expression of this protein decreases with tumor progression. In our study, expression of Smac/DIABLO protein was significantly lower in pT2 and pT3 tumors comparing to pT1, which confirms this hypothesis. However, no correlation with other staging features such as pathological nodal status (in our group of patients no metastatic disease on diagnosis where observed) was observed. In other studies, correlation with Smac/DIABLO was not so evident. In renal cell carcinoma, expression of Smac/DIABLO protein was higher in I/II stage comparing to stage III/IV (96% vs. 50%)[13]. In other studies conducted in RCC, no correlation between Smac/DIABLO and stage of disease was observed at the mRNA level (8,12). In cervical cancer, expression of Smac/DIABLO mRNA was not linked with tumor stage (17).

An age less than 35 years old has been indicated as an independent unfavorable prognostic factor in breast cancer (2,18). Since in our study, the youngest patient was 41, we stratified the patients into two groups: under 50 years old and over 50 years old . We reported a higher expression of Smac/DIABLO protein in the subgroup of patients under 50 years old. Further investigations are needed with larger group of patients to verify this observation.

In other clinicopathological features of the study group, diffuse cancer infiltration in the vicinity of primary tumors inversely correlated with Smac/DIABLO expression. Additionally cancer embolus in minor blood and lymphatics vessels showed a tendency toward lower expression of Smac/ DIABLO. Both results confirmed the hypothesis that more advanced tumors tend to decrease Smac/DIABLO protein expression.

To verify whether the presence of Smac/DIABLO protein induces apoptosis in breast cancer, we assessed the expression of caspase-3 protein, which directly induces nuclear changes during apoptosis. In our study, expression of Smac/DIABLO protein strongly correlated with caspase-3 protein, confirming the pro-apoptotic activity of Smac/DIABLO protein in this malignancy.

The results of our study suggest that Smac/DIABLO protein may play an important role in breast cancer development. The potential prognostic value must be verified in further studies encompassing treatment outcomes.

Acknowledgments. This work was supported by the Polish Ministry of Science and Higher Education from the financial resources allocated for Science for 2008-2011 (Research Grant No. NN 403182234). We thank mgr Edward Lowczowski, Foreign Language Teaching Center, Medical University of Lodz for his assistance with the English language.

References

[1] OLOFIN I, OLOPADE OL. Eliminating global disparities in breast cancer care through clinical research. In: ASCO Educational Book. Alexandria, Virginia 2009: 681-684.

[2] GOLDHIRSCH A, WOOD WC, GELBER RD, COATES AS, THURLIMANN B et al. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer. Ann Oncol 2007; 18: 1133-1144. doi:10.1093/annonc/mdm271

[3] HANAHAN D, WEINBERG RA. The hallmarks of cancer. Cell 2000; 100: 57-70. doi:10.1016/S0092-8674(00)81683-9

[4] HEGDE R, SRINIVASULA SM, ZHANG Z, WASSELL R, MUKATTASH R et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem 2002; 277: 432-438. doi:10.1074/jbc.M109721200

[5] MARTINS LM, IACCARINO I, TENEV T, GSCHMEISSNER S, TOTTY NF et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem 2002; 277: 439-44.

[6] VERHAGEN AM, SILKE J, EKERT PG, PAKUSCH M, KAUFMANN H et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem 2002; 277: 445-454.

[7] LEAMAN DW, CHAWLA-SARKAR M, VYAS K, REHEMAN M, TAMAI K et al. Identification of X-linked inhibitor of apoptosis-associated factor-1 as an interferon-stimulated gene that augments TRAIL Apo2L-induced apoptosis. J Biol Chem 2002; 277: 28504-11. doi:10.1074/jbc.M204851200

[8] YAN N, SHI Y: Mechanisms of apoptosis through structural biology. Annu Rev Cell Dev Biol 2005; 21: 35-56. doi:10.1146/annurev.cellbio.21.012704.131040

[9] YOO NJ, KIM HS, KIM SY, PARK WS, PARK CH et al. Immunohistochemical analysis of Smac/DIABLO expression in human carcinomas and sarcomas. APMIS 2003; 111: 382-388. doi:10.1034/j.1600-0463.2003.t01-1-1110202.x

[10] ADRAIN C, CREAGH EM, MARTIN SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J 2001; 20: 6627-6636. doi:10.1093/emboj/20.23.6627

[11] EHEMANN V, SYKORA J, VERA-DELGADO J, LANGE A, OTTO HF. Flow cytometric detection of spontaneous apoptosis in human breast cancer using the TUNEL-technique. Cancer Lett 2003; 194: 125-131. doi:10.1016/S0304-3835(03)00054-5

[12] KEMPKENSTEFFEN C, FRITZSCHE FR, JOHANNSEN M, WEIKERT S, HINZ S et al. Down-regulation of the pro-apoptotic XIAP associated factor-1 (XAF1) during progression of clear-cell renal cancer. BMC Cancer 2009; 9: 276. doi:10.1186/1471-2407-9-276

[13] MIZUTANI Y, NAKANISHI H, YAMAMOTO K, LI YN, MATSUBARA H et al. Downregulation of Smac/DIABLO expression in renal cell carcinoma and its prognostic significance. J Clin Oncol 2005; 23: 448-454. doi:10.1200/JCO.2005.02.191

[14] BAO ST, GUI SQ, LIN MS: Relationship between expression of Smac and Survivin and apoptosis of primary hepatocel-

lular carcinoma. Hepatobiliary Pancreat Dis Int 2006; 5: 580-583.

[15] PLUTA A, WRZESIEN-KUS A, CEBULA-OBRZUT B, WOLSKA A, SZMIGIELSKA-KAPLON A et al. Influence of high expression of Smac/DIABLO protein on the clinical outcome in acute myeloid leukemia patients. Leuk Res. doi:10.1016/j.leukres.2009.11.030, 2010. doi:10.1016/j.leukres.2009.11.030

[16] REN Y, AKYUREK N, SCHLETTE E, RASSIDAKIS GZ, MEDEIROS LJ. Expression of Smac/DIABLO in B-cell non-Hodgkin and Hodgkin lymphomas. Hum Pathol 2006; 37: 1407-1413. doi:10.1016/j.humpath.2006.06.006

[17] ESPINOSA M, CANTU D, LOPEZ CM, DEL LA GARZA JG, MALDONADO VA, MELENDEZ-ZAJGLA J. SMAC is expressed de novo in a subset of cervical cancer tumors. BMC Cancer 2004; 4: 84. doi:10.1186/1471-2407-4-84

[18] FREDHOLM H, EAKRER S, FRISELL J, HOLMBERG L, FREDRIKSSON I et al. Breast cancer in young women: poor survival despite intensive treatment. PLoS On. doi: 10.1371/journal.pone.0007695, 2009.