Immunoexpression of DIABLO, AIF and Cytochrome *c* in Gastric Adenocarcinoma Assessed by Tissue Microarray

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Abstract. *The aim of this study was to analyze the immunoexpression of (Smac) DIABLO, AIF, cytochrome c, Ki-67 and cleaved caspase-3 in gastric cancer. A tissue microarray (TMA) paraffin block was constructed using gastric adenocarcinoma tissue and adjacent normal adjacent mucosa from 87 patients who had not previously undergone radiotherapy or chemotherapy. Immunohistochemistry was used to evaluate the protein levels. Samples were positive for (Smac) DIABLO in 37 (45.6%) and 37 (46.8%), for AIF in 31 (36.9%) and 36 (45.6%), for cytochrome c in 60 (68.9%) and 44 (54.4%), for Ki-67 in 63 (72.4%) and 52 (61.9%) and for cleaved caspase-3 in 21 (24.1%) and 3 (3.4%) cases of tumor and adjacent normal tissues, respectively. Our results suggest that increased expression of Ki-67 and cleaved caspase-3 could contribute to carcinogenesis. The expression of these proteins indicates an attempt of cells to maintain tissue homeostasis.*

Gastric carcinoma is a frequent cause of morbidity and mortality in populations of Asian countries. Brazil has an intermediate incidence. Estimates for 2012 for the south-eastern region report an approximate incidence of 14.84-18.96 men and 8.17-9.66 women per 100,000 habitants (1).

Gastric carcinogenesis occurs in multiple cumulative stages, including multiple genetic and epigenetic alterations in oncogenes, tumour-suppressor genes, cell-cycle regulators, cell-adhesion molecules, DNA repair genes, and genetic instability, as well as telomerase activation (2). Given this context, several metabolic pathways of cellular proliferation, DNA repair and apoptosis have been studied (3-6).

Apoptosis (programmed cell death) is an efficient physiological mechanism for cell suicide that is important for growth and cell differentiation processes in multicellular animals, controlling the number of cells and organ size (7). It is activated by two pathways: the cell death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. Both pathways promote the activation of the caspase cascade, a family of cysteine proteases that constitute the central regulatory mechanism of death by apoptosis. Secondary mitochondrial activation of caspases (Smac; now known as DIABLO) is a mitochondrial protein released in the cytosol, similarly to cytochrome *c*, after apoptotic stress. DIABLO is located in the intermembrane mitochondrial space as a mitochondrial precursor protein and is activated by the removal of the N-terminal sequence. Yoo *et al.* suggested that lack of DIABLO expression in cancer cells may inhibit apoptosis, thereby promoting their survival (8).

Apoptosis-inducing factor (AIF) is a flavoprotein that can oxidize NADH and NADPH *in vitro* and may participate in the detoxification of reactive oxygen species (9). The AIF oxidoreductase activity accounts for resistance to oxidative stress and maintenance of transformated colonic cancer cells (10). AIF is *N*-terminally anchored to the inner mitochondrial membrane and must be liberated from its membrane anchor prior to being released into the cytosol. On the other hand, AIF causes caspase-independent chromatin condensation and DNA fragmentation, demonstrating that it plays an important role in apoptosis (11-13). Somatic mutation of *AIF* is considered a rare event in common human cancer (14).

Cytochrome *c* is a carrier of electrons from the mitochondrial inner membrane protein complex III to complex IV and is essential for the generation of the mitochondrial membrane potential that drives the formation of ATP. In human and other vertebrate cells, cytochrome *c* is also a central apoptotic effector. Cytochrome *c* release into the cytosol is particularly associated with activation of the intrinsic apoptosis pathway, which responds to intracellular

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stimuli such as DNA damage, lineage information and oncogene activation. Once in the cytosol, cytochrome *c* binds the adaptor protein apoptotic protease-activating factor-1 (Apaf-1) and assembles the apoptosome complex, causing recruitment and activation of the initiator caspase-9 (11, 15). The subsequent proteolytic cascade effects the morphological changes that define apoptosis including cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation of cells into apoptotic bodies that can be rapidly cleared. During apoptosis, cytochrome *c* is released from mitochondria, along with the protein DIABLO.

Gastric cancer is generally poorly responsive to chemotherapy and radiotherapy, suggesting that these tumors are intrinsically resistant to the apoptosis-inducing effects of anticancer drugs and X-irradiation. There are few studies on DIABLO, AIF and cytochrome *c* proteins in gastric cancer tissues. In this study, an immunohistochemical method was used to characterize the expression of Smac/DIABLO, AIF and cytochrome *c* in tissues of gastric adenocarcinoma originating from Brazilian patients and to verify what role they play in apoptosis.

Patients and Methods

Tissues. Eighty-seven gastric adenocarcinoma tissues, previously classified as either intestinal or diffuse type according to Lauren were retrieved from the files of Department of Pathology of Sao Paulo Federal University. Ethical approval for this study was granted by the local Ethics Committee (Resolution n. 196 of the National Health Council).

Tissue Microarray (TMA). Histological sections of 4 μm were cut from each block and stained by hematoxylin-eosin. The slides were evaluated for diagnostic confirmation and re-evaluation of the histopathological findings, including the selection of sites for the removal of cylindrical cores used in TMA block construction.

TMA blocks were constructed using Beecher™ equipment (Beecher Instruments, Silver Spring, MD, USA) according to the manufacturer's instructions, in the following stages: i) The selected area in the respective paraffin block was marked; ii) a cylindrical core was created in the receptor block using the apparatus; iii) a 1-mm cylinder of tissue was extracted from the area of interest; iv) the cylindrical tissues obtained from the donating block were transferred to the core in the receptor block; v), New core positions were created in the receptor block, separated by fractions of mm such that a collection of tissue samples was created following the matrix arrangement; vi) the quality of the block was assessed before storage. To guarantee adhesion of the TMA block slices on the slides, an adhesive tape system (Instrumedics Inc, Hackensak, NJ, USA) was used.

Immunohistochemistry. Sections of 3 μm mounted on 3-aminopropylotrimetoxy silane- (Sigma Aldrich, St. Louis, USA) coated slides were de-waxed in xylene, and taken through ethanol to water to rehydrate. For antigen retrieval, slides were placed in 0.01 M citrate-buffer (pH 6.0) and heated in a steamer for 30 min. Endogenous peroxidase activity was blocked by incubating the sections in a solution of 3% hydrogen peroxide for 20 min at room temperature. After these procedures, the sections were incubated with a DIABLO mouse monoclonal antibody (1:200) (Cell Signaling), AIF rabbit polyclonal (H-300) (1:400) (Santa Cruz Biotechnology, Inc. USA), cytochrome *c* goat polyclonal antibody (C-20) (1:500) (Santa Cruz Biotechnology, Inc.), anti-cleaved caspase-3 rabbit polyclonal antibody (AP1027) (1:100) Calbiochem-Merck KgaA), Ki-67 mouse monoclonal antibody (clone MIB-1) (1:100) Dako North America, Inc., at 4˚C overnight. The sections were washed (PBS) and allowed to react with LSAB+ System-HRP (Biotinylated Link Universal) (Streptavidin-HRP) (Dako North America Inc.) for 30 min. Finally, staining was carried out using Liquid DAB+substrate chromogen system (Dako North America, Inc.) lightly counterstaining with Harris hematoxylin, and sections were then coverslipped with Entellan (Sigma). Negative and positive controls were ran simultaneously. Positive controls were represented by colonic adenocarcinoma tissues. Negative controls were made by omitting the primary antibody.

A cytoplasmic expression pattern for DIABLO, AIF, cytochrome *c* and cleaved caspase-3 was observed, while a nuclear expression pattern was seen for Ki-67. These patterns were analyzed according to distribution and intensity criteria. A numerical scoring system with two categories was used to assess protein expression (16). Category A documented the number of immunoreactive cells as 0 or negative (no immunoreactive cells or <10% immunoreactivity), 1 (10 to <25%), 2 (25 to <50%) and 3 (>50% immunoreactivity). Category B documented the intensity of the immunostaining as 0 or negative, 1 (no or weak immunostaining), 2 (moderate) and 3 (strong). The values of categories A and B were multiplied, resulting in an immunoreactive score ranging from 0 to 9, with 0-3 considered negative and 4-9 positive. To evaluate the expression of Ki-67 protein, Eclipse 80i-Nikon was used. Cells were counted in fields showing a more intense reaction ('hot-spots'); 200 cells were counted at ×400 and the percentage of immunostained cells was recorded (17). Representative areas of gastric adenocarcinoma tissues and adjacent normal gastric mucosa were captured using a Sony camera at ×400.

Statistical analysis. The relationships between expression of DIABLO, AIF, cytochrome *c*, cleaved caspase-3 and Ki-67 and various clinicopathological findings were evaluated using Chi-square test. Kaplan Meier survival curves were constructed to assess whether expression of DIABLO, AIF, cytochrome *c*, cleaved caspase-3 and Ki-67 had any effect on overall survival of patients with gastric cancer. A *p*-value less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 15.0 for Windows.

Results

Clinicopathological data from the patients with gastric cancer are summarized in Table I. Fifty-one cases were men and 36 cases were women, with an overall mean age of 61.3 years (range=36 to 85 years). The median follow-up period was 20 months (range=1 day to 77 months). Patient survival was classified as no evidence of disease (n=2), died of disease (n=64), alive with disease (n=16), and lost to follow-up (n=10). The majority of cases (66.7%) had stage III disease. The histological subtypes included 59 intestinal adenocarcinomas and 28 diffuse adenocarcinomas.

Table I. *Clinicopathological variables of samples*.

Variable		N (%)
Gender	Male	51 (58.6)
	Female	36 (41.4)
Age	≤50 years	21 (24.1)
	>50 years	66 (75.9)
Clinical stage	I	13 (14.9)
	II	15 (17.2)
	III	58 (66.7)
	IV	01 (1.1)
	Early (I)	13 (15.0)
	Advanced (II, III, IV)	74 (85.0)
Follow-up	Alive with disease	13 (14.9%)
	Died from cancer	64 (73.6%)
	Lost	10 (11.5%)
Lymph node status	Positive	63 (72.4)
	Negative	24 (27.6)
Surgery	Total gastrectomy	40 (46.0)
	Partial gastrectomy	47 (54.0)
Tumor size	≤3 cm	21 (24.1)
	>3 cm	66 (75.9)
Laurén classification	Intestinal	59 (67.8)
	Diffuse	28 (32.2)
Site	Cardia	29 (33.3)
	Antrum	38 (43.7)
	Corpus	20 (23.0)

Table II. *Immunohistochemical analysis of DIABLO, AIF, cytochrome c, cleaved caspase-3 and Ki-67 in the tumor and the normal adjacent mucosa*.

Protein	Tumor	Normal gastric mucosa	*p*-Value
	N (%)	N (%)	
DIABLO			
Negative	44 (54.4%)	42 (53.2%)	0.991
Positive	37 (45.6%)	37 (46.8%)	
AIF			
Negative	53 (63.2%)	43 (54.6%)	0.335
Positive	31 (36.9%)	36 (45.6%)	
Cytochrome *c*			
Negative	27 (30.9%)	37 (45.6%)	0.073
Positive	60 (68.9%)	44 (54.4%)	
Cleaved caspase-3			
Negative	66 (75.9%)	84 (96.6%)	0.001
Positive	21 (24.1%)	03 (3.4%)	
Ki-67			
Negative	24 (27.6%)	32 (38.1)	0.019
Positive	63 (72.4%)	52 (61.9)	

The cytoplasmic expression pattern for DIABLO, AIF, cytochrome *c*, cleaved caspase 3 and the nuclear expression pattern for Ki-67 was recorded (Figure 1A-E, respectively). The tumor and the adjacent normal tissues were positive for DIABLO in 37 (45.6%) and 37 (46.8%), for AIF in 31 (36.9%) and 36 (45.6%), for cytochrome *c* in 60 (68.9%) and 44 (54.4%), for Ki-67 in 63 (72.4%) and 52 (61.9%) and for cleaved caspase-3 in 21 (24.1%) and 3 (3.4%) cases, respectively (Table II). A positive correlation between cytochrome *c* and AIF and DIABLO and cleaved caspase-3 were observed. (Table III). There was no association of biomarkers with survival.

Discussion

Caspases are effector enzymes for the apoptotic process and their activity is subject to rigorous molecular control by IAPs, which in turn are controlled by molecular inhibitors. DIABLO is the principal apoptosis inhibitor of IAPs and the most studied (18, 19). Besides DIABLO, AIF and cytochrome *c* are also pro-apoptotic proteins.

In an attempt to understand gastrointestinal carcinogenesis, several studies have investigated abnormal expressions of DIABLO, AIF and cytochrome *c* in normal gastric mucosa and in pre-neoplastic lesions (20, 21).

In this report, we used TMA technology and immunohistochemistry to detect the expression of DIABLO, AIF and cytochrome *c* proteins in human gastric adenocarcinoma and their corresponding normal gastric mucosa, and to explore the distribution of these proteins in human gastric adenocarcinoma and their biological significance.

TMA technology presents a method for serial analyses of protein expression in hundreds of tissue specimens. In general, this method has been proven to be highly effective for the analysis of protein alterations in different stages of one particular tumor (22, 23). In addition, immunohistochemistry has the advantage of allowing protein expression and its location to be determined.

During apoptosis, DIABLO is released along with cytochrome *c* from mitochondria into the cytosol to promote the activation of caspase-3. DIABLO interacts with IAPs, and then inhibition of IAPs to caspases is relieved, so that caspases are activated to exert their biological activities.

In this study, the expression of DIABLO was noted in 37 (45,6%) tumors and in 37 (46.8%) normal adjacent mucosa. No difference was found in the expression of this protein in tumor tissues compared to normal mucosa. On the other hand, Yoo *et al*. observed immunoreactivity for DIABLO in 70% of advanced gastric carcinomas (8). Expression of cleaved caspase-3 was increased in tumor tissues when compared to normal adjacent mucosa. This result suggests that the expression of DIABLO is locked and it is not eliminating the inhibitory effect of IAPs on caspases.

Shibata *et al*. (24) showed that mRNA expression of *DIABLO* was higher in gastric adenocarcinomas than in non-neoplastic gastric mucosa. On the other hand, Zhao *et al*. (20) showed that mRNA expression of *DIABLO* was lower

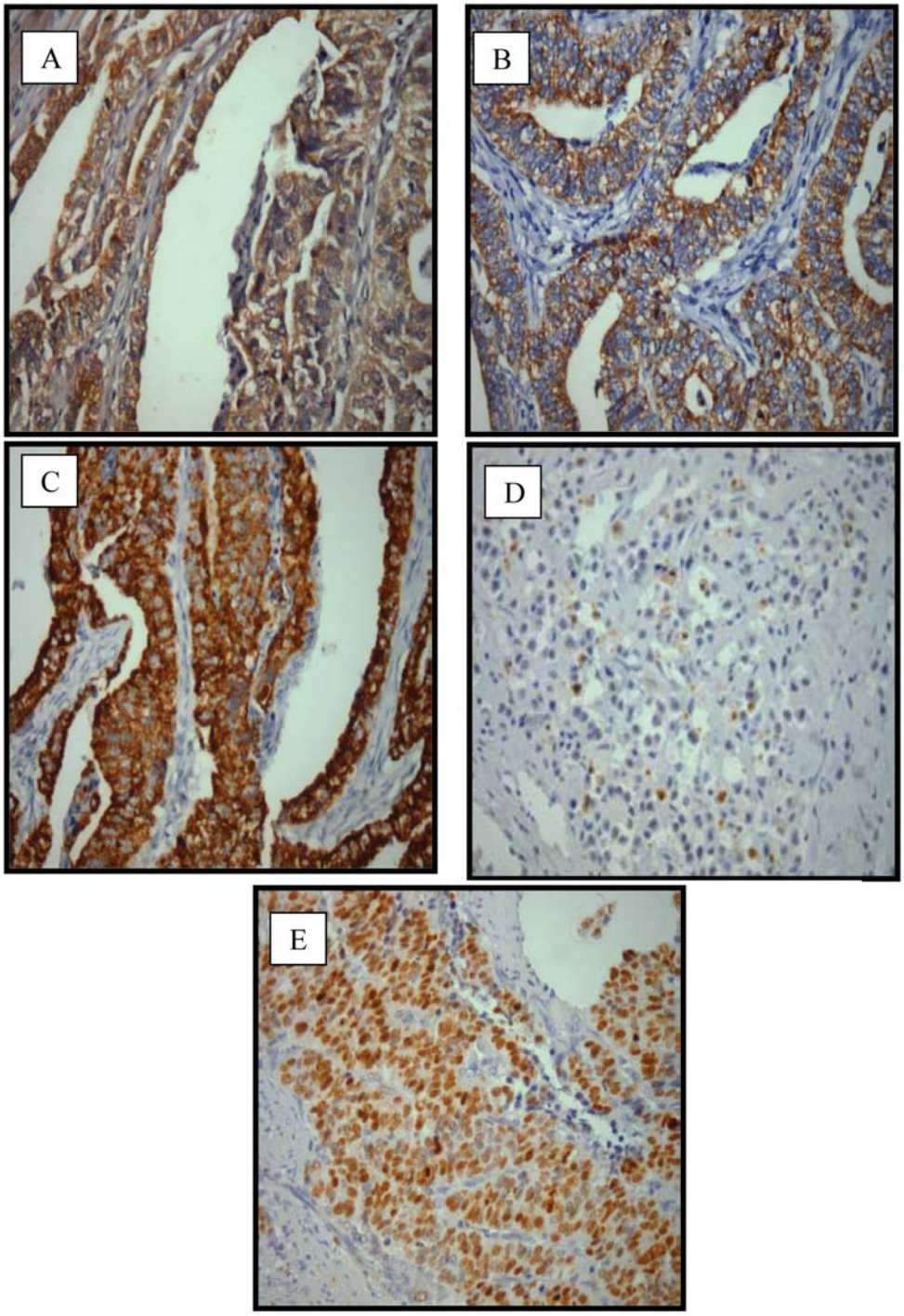

Figure 1. *Immunohistochemical analysis of gastric adenocarcinoma tissues. The cytoplasmic expression pattern for DIABLO (A), AIF (B), cytochrome c (C), cleaved caspase-3 (D) and nuclear expression pattern for Ki-67 (E) is observed. Magnification ×400.*

in gastric carcinomas than in normal gastric tissues. There is little literature concerning DIABLO and gastric cancer, and the results available are very conflicting.

The major function of AIF is the induction of cell death but it has another function that is the maintenance of cellular survival that is essential for cancer development. AIF has an oxidoreductase activity that is required for cell survival and cancer cells without this capability may easily die.

Daugas *et al*. (12) reported AIF protein expression in 65 human cancer cell lines of various origins and observed that

Table III. *Correlation between DIABLO, AIF, cytochrome c, Ki-67 and cleaved caspase-3 protein expression in the tissues of gastric adenocarcinoma.*

		Cytochrome *c*	AIF	Smac/DIABLO	Ki-67	Cleaved caspase-3
Cytochrome *c*	Correlation	1	0.342	0.158	0.027	0.112
	p-value		0.001	0.160	0.805	0.303
AIF	Correlation		1	–0.107	–0.166	0.063
	p-value			0.346	0.130	0.569
Smac/DIABLO	Correlation			1	0.235	–0.003
	p-value				0.035	0.979
Ki-67	Correlation				1	0.125
	p-value					0.248

colon cancer cell lines strongly expressed AIF. AIF was also found to be widely expressed in normal human tissues, including colonic enterocytes. The wide expression of AIF in both cancer and normal tissues may indicate that AIF has a vital function that is common to cancer and normal tissues.

Pro-apoptotic stimuli induce the translocation of AIF to the nucleus, mediating a cell death that is completely independent of cytochrome *c* release, caspase activation and observable mitochondrial damage (25, 26). Pro-apoptotic stimuli induce the release of AIF to the cytosol after cytochrome *c* is released suggesting that AIF translocation from mitochrondria to the nucleus may be dependent, in part, on prior caspase activation induced by cytochrome *c* (27). However, research involving AIF and gastric cancer is very scarce.

In this study, the expression of AIF was noted in 31 (36.9%) tumors and in 36 (45.6%) normal adjacent mucosa. No difference was found in the expression of this protein in tumor tissues compared to normal mucosa. There were no associations between AIF and other clinical and pathological variables, and no correlation between patient survival and the expression of this protein was observed. These results suggest that AIF participates in the apoptotic mechanism to a lesser extent during tumor progression.

The ability of cytochrome *c* to initiate apoptosis depends on numerous cellular factors, including the intracellular redox environment. Oxidized but not reduced cytochrome *c* activates caspases and promotes apoptosis (28-30).

BAX and BAK, pro-apoptotic members of the BCL-2 family, actively induce cytochrome *c* release from mitochondria within cells prior to caspase activation (31).

Increased expression of cytochrome *c* was observed in 60 (68.9%) of the tumors, compared to 44 (54.4%) of the normal adjacent mucosa. No difference was found in the expression of this protein in tumor tissues compared to normal mucosa. Cytochrome *c* was more frequently expressed in the intestinal, compared to the diffuse type ($p<0.018$). Perhaps this result is due to the greater number of intestinal-type tumors. There were no associations between cytochrome *c* expression and other clinical and pathological variables, and no correlation between patient survival and the expression of this protein was observed.

Expression of the Ki-67 protein is considered to be an important prognostic factor in malignant tumors. Barrezueta *et al*. (16) studying the same population of gastric cancer found increased cell proliferation in tumor tissues compared to adjacent normal mucosa. Oshima *et al*. observed higher expression of Ki-67 in intestinal type-compared with diffuse type adenocarcinomas (32). In another study by Oshima *et al*., which determined the rate of cell proliferation (by Ki-67) in gastric and colonic cancer, a wide variation in the percentage and intensity of Ki-67 expression was noted. The expression was higher in gastric carcinomas (32). Increased expression of Ki-67 was observed in 63 (72.4%) of the tumors, compared to 52 (61.9%) of the normal adjacent mucosa. There was marked difference in the expression of this protein in the tumor tissues compared to normal mucosa. In this study, we found an association between the expression of the Ki-67 protein and tumor size with large tumors showing an increased Ki-67 rate.

In this study, the expression of cleaved caspase-3 was noted in 21 (24.1%) tumors and in 3 (3.4%) normal adjacent mucosa. There was marked difference in the expression of this protein in tumor tissue compared to normal mucosa. The decreased expression of cleaved caspase-3 is probably due to IAPs preventing caspase-3 activation and trigger cell death. This suggests that caspase-3 is not implicated in gastric carcinogenesis. Zheng *et al*. (33) also found that the caspase-3 protein was more strongly expressed in intestinal type cancers than in the diffuse type (34).

A correlation was demonstrated between cytochrome *c* and AIF ($p<0.001$) and DIABLO and Ki-67 ($p<0.035$) in adenocarcinomas. This observation suggests that cytochrome *c* and AIF work together, triggering the mechanism of apoptosis. However, the cellular proliferation exceeds the cellular death, as indicated by Ki-67 and DIABLO.

Upper gastrointestinal adenocarcinomas are typically characterized by poor response to therapy and low survival rates (35). In this study, the Kaplan Meier curves showed no significant differences regarding patient survival between the

groups presenting positive or negative immunoexpression of Smac/DIABLO, AIF or cytochrome *c*.

Our results suggest that increased expression of Ki-67 and cleaved caspase-3 could contribute to carcinogenesis. The expression of these proteins indicates an attempt of cells to maintain tissue homeostasis. Further studies are needed to address this important mechanism in gastric adenocarcinomas.

Conflicts of Interest

None declared.

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