Expression of Bax protein in gastric carcinomas. A clinicopathological and immunohistochemical study

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Abstract

Background and study aims : **Reduced Bax protein expression has been shown to be a negative prognostic factor in patients with breast, ovarian, colorectal, esophageal and pancreatic cancer. Our aim was to immunohistochemically study Bax protein expression in gastric carcinomas and correlate its expression with clinicopathological parameters and prognosis.**

Patients and methods : **Immunohistochemistry was performed, using a monoclonal antibody against bax, in paraffin-embedded tumor specimens from 47 cases of gastric cancer.**

Results : **Positive staining for the Bax protein was found in 20/47 (42.4%) adenocarcinomas examined. Negative Bax protein expression in tumour cells was correlated with lymph node metastasis ($P < 0.05$), and degree of differentiation ($p<0.05$). Univariate analysis showed that the variables with a significant negative impact on survival were : high TNM tumour stage, depth of penetration in the gastric wall, lymph node involvement, and Bax protein expression. Multivariate analysis showed that the only variable with an impact on survival was Bax protein expression ($p<0.05$, Relative Risk : 3.34). Kaplan-Meier curves showed that the 5-year survival was 36.8% in cases with positive compared with 16% in cases with negative Bax protein expression ($p=0.0427$).**

Conclusion : **Negative Bax expression in gastric cancer is associated with de-differentiation, lymph node metastases, and poor clinical prognosis. Bax protein expression might play an important role in the development and phenotypic differentiation of gastric carcinomas and tumor progression.** (Acta gastroenterol. belg., **2007**, 70, **285-289**).

Key words : bax, gastric cancer, immunohistochemistry, prognosis, apoptosis.

Introduction

Gastric cancer is still the second most common cause of cancer-related death in the world (1). It is generally believed that gastric cancer evolves through a multistep progression from chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and subsequently to cancer. In particular, atrophic gastritis and intestinal metaplasia are considered to be the precursors of gastric cancer, especially for the intestinal type of gastric malignancy (2).

Apoptosis, or programmed cell death, is a widespread process used to eliminate unwanted or damaged cells from multicellular organisms. Several recent studies have documented a possible role for apoptosis in the development or progression of malignant neoplasms (3). Apoptosis plays an essential role in maintaining tissue integrity within the gastric epithelium. Failure of the apoptotic process predisposes an increased survival of cells with DNA damage from which gastric cancer may arise (4-5).

Two major apoptotic pathways have been identified ; the death receptor pathway and the mitochondrial pathway (6-7). The mitochondrial pathway is regulated by members of the Bcl-2 protein family (8). Bax belongs to the Bcl-2 family of proteins, and is a key player in apoptosis, accelerating cell death after an apoptotic stimulus. Inactivation of Bax during tumorigenesis may contribute to tumor progression by enhancing escape of damaged cells from apoptosis (9). Reduced Bax expression has been shown to be a negative prognostic factor and correlates with a shorter overall survival in patients with breast, ovarian, colorectal, esophageal and pancreatic cancer (8).

A previous study has shown that the suppression of Bax and overexpression of Bcl-2 protein is an early event in gastric tumorigenesis, before gastric dysplastic changes occur (10). However, little is known about its expression and its relationship with the biological behavior of human gastric cancer.

To determine whether the pre-apoptotic gene Bax plays a role in the regulation of apoptosis in gastric carcinoma, an immunohistochemical analysis of Bax protein expression in gastric carcinoma was performed. Its expression was correlated with clinicopathological parameters and prognosis.

Patients/materials

Forty seven cases of gastric carcinomas (either surgically resected or biopsied with large biopsy forceps) were collected from the files of the Department of Pathology within our hospitals. Out of these 47 subjects, 31 were male and 16 female. The mean age was 69 years

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from an age range of 45-87 years. All blocks were fixed in 10% formalin and embedded in paraffin. Serial sections were cut from each block in 4 µm, stained with haematoxylin and eosin and confirmed pathologically. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of each hospital.

Utilising the International Tumor-Node-Metastasis (TNM) staging system, tumours were considered : stage IB - 6 (12.7%), stage II - 10 (21.2%), stage IIIA - 9 (19.1%), stage IIIB - 3 (6.3%), and 19 (40.7%) stage IV. Histological grade was assessed following the World Health Organization (WHO) criteria. Four (8.5%) of the tumors were well differentiated, 18 (38.2 %) were moderately differentiated, and 25 (53.3 %) were poorly differentiated.

The EnVision System and the monoclonal antibodies for BAX (DAKO, Glostrup, Denmark ; dilution 1:500) were used for immunohistochemical analysis. In summary, 5-µm histological sections were dewaxed in xylene and rehydrated using a graded alcohol series. The sections were immersed in 10 µmol Tris and 0.5 mol/L ethylenediamine tetraacetic acid (EDTA) (pH 9.0), and microwaved twice for 5 min each time. Subsequently, the sections were incubated with 0.3% H2O2 for 30 min to block endogenous peroxidase activity. They were then incubated with the primary antibodies overnight at 4°C. Non-specific binding was blocked by incubating the sections for 30 min with blocking solution (Dako). Detection was carried out using the EnVision System kit (Dako), with diaminobenzidine as the chromogen. Counterstaining was performed with Harris hematoxylin.

For each section, 10 high-power fields were chosen randomly, and a total of 1,000 cells were evaluated by two different observers. The observers did not have any prior knowledge of patient outcome or tumor characteristics. Bax expression patterns were graded as follows : 0 - completely negative, 1 - less than 10% of cells stained, 2 - < 20% of cells stained, 3 - 20-50% of cells stained, and 4 - > 50% of cells stained. Lymphocytes and small vessels were used as positive controls of Bax immunoreactivity. Negative controls were performed by omitting the primary antibody.

Statistical analysis

Statistical analysis for group differences was performed using Chi-square or Fisher's exact test. The Cox proportional hazards regression model was used to assess the relative influences of the following covariates : age, sex, histologic type, TNM stage, depth of gastric wall penetration, lymph node involvement, degree of differentiation, and positive/negative Bax protein expression. Univariate, multivariate (proportional hazard regression model), as well as Kaplan-Meier survival analysis were performed. For all statistical tests, a *P* value of less than 0.05 was considered significant.

Table 1. — **Bax immunoreactivity in gastric carcinomas**

20/47 carcinomas stained positively for Bax	Bax immunoreactivity (% of cells stained positively)			
	< 5%	5-20%	20-50%	> 50%
	10 (50%)	6 (30%)	3 (15%)	1 (5%)

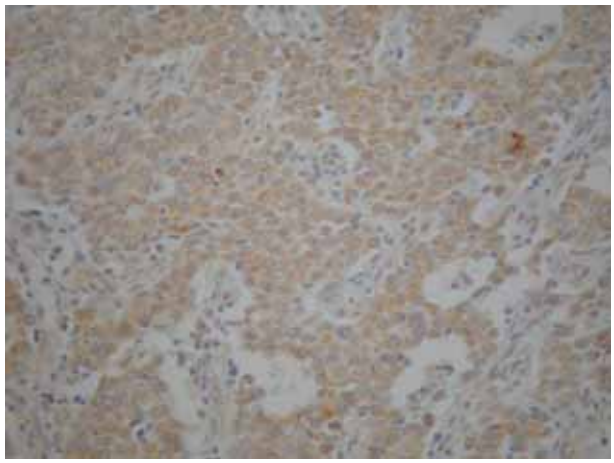

Fig. 1. — Gastric cancer stained positively for bax protein

Results

Positive immunohistochemical staining for the Bax protein was found in 20 (42.4%) of the 47 adenocarcinomas examined (Table 1). The Bax protein immunoreactivity appeared brown and was mainly located in the cytoplasm. In 10/20 cases (50%) protein expression was found in < 5% of tumour cells ; of which 6/10 cases found protein expression in 5-20% of tumour cells. In 3/10 cases it was evident in 20-50% of tumour cells and in one case in > 50% of tumour cells (Fig. 1).

The distribution of tumors with negative and those with positive Bax protein expression were examined (Table 2). There were no significant differences found for sex, age, penetration through gastric wall, and TNM stage. However, statistical analysis demonstrated a significant association between negative Bax protein expression and lymph node involvement and histologic type of tumor.

Bax expression was negative in 17/24 (70.8%) cases of intestinal type, and in 10/23 (43.4%) cases of diffuse type gastric adenocarcinomas (p = ns). Negative Bax protein expression in tumor cells was correlated with lymph node metastasis (P < 0.05). 24/27 (88.8%) cases with lymph node involvement had negative Bax protein expression, while 7/20 (35%) cases without lymph node metastases had positive Bax protein expression. Bax protein expression was negative in 22/25 (88%) cases of poor-differentiated carcinomas, 14/18 (77.7%) cases of moderately-differentiated and in 1/4 (25%) cases of well-differentiated gastric adenocarcinomas (P < 0.05, poor vs well differentiated).

Table 2. — **Correlation between Bax protein expression and clinicopathological parameters of gastric carcinomas**

Variable	*Total*	*BAX -*	*BAX +*	*P*
Age				
< 70	24	12	11	NS
> =70	23	14	9	
Sex				
Male	31	18	13	NS
Female	16	9	7	
Type				
Intestinal	24	17	7	NS
Diffuse	23	10	13	
Differentiation				
Poor	25	19	6	p < 0.05*
Moderate	18	7	11	NS**
Well	4	1	3	
Penetration through gastric wall				
T1-2	7	4	3	NS
T3	16	10	6	
T4	24	13	11	
Lymph node involvement				
Positive	27	24	3	p < 0.05***
Negative	20	13	7	
TNM Stage				
IB	6	3	3	NS
II	10	9	1	
IIIA	9	7	2	
IIIB	3	2	1	
IV	19	16	3	

*P < 0.05 x^2 = 4.19, poor vs well differentiated gastric carcinoma, **NS, moderate vs well differentiated gastric carcinomas, ***P < 0.05, x2 = 3.90, presence vs absence of lymph node involvement.

Analysis of survival was carried out in 44 of the initial 47 cases (3 patients were lost to follow-up). During follow-up evaluation (median, 54 months), 33 (75%) patients died of gastric cancer, and there were no deaths from other causes. Five-year overall survival was 100% in stage IB patients, 37.5% in stage II patients, 22% in stage IIIA patients, 0% in stage IIIB patients, and 0% in stage IV patients.

The results of Cox regression analysis of survival in relation to clinicopathological findings are reported in Table 3. Univariate analysis showed that the variables with a significant negative impact on survival were : high TNM tumour stage, depth of penetration in the gastric wall, lymph node involvement, and Bax protein expression. Multivariate analysis showed that the only variable with an impact on survival was bax protein expression (p < 0.05, Relative Risk : 3.34, CI : 1.0068-11.1282).

Kaplan-Meier curves showed that the five-year survival was 36.8% in cases with positive Bax protein expression compared with 16 % in cases with negative Bax protein expression (p = 0.0427). The median survival time in the two groups was 48 and 24 months respectively (Fig. 2).

Discussion

Genetic control of apoptosis and cell survival plays a crucial role in tumor growth. Among the molecules related to the apoptotic process, the Bcl-2 family proteins are important critical regulators in a variety of physiological and pathological contexts (8). Bcl-2 and Bax are members of the Bcl-2 family. Bcl-2 prevents cell death through a variety of mechanisms, whereas overexpression of Bax protein increases the susceptibility of cells to apoptosis. Bax is localised in the cytoplasm and translocates to mitochondria in response to apoptotic stimuli. It promotes cell death by inducing the formation of ion-permeable pores that disrupt the mitochondrial membrane barrier, resulting in the release of cytochrome C to the cytosol. In addition, Bax forms heterodimers with Bcl-2, with one monomer antagonizing the function of the other. Bax is also a crucial factor linking the regulation of mitochondrial physiology and apoptosis to the surveillance of DNA integrity by p53 (8). Several lines of evidence indicate that the Bax pro-apoptotic protein plays a tumour suppressor role ; whilst the integrity of Bax may have important consequences in the progression of epithelial tumours by determining the outcome of chemotherapeutic regimens (11). However, little exists in the literature about Bax protein expression and its relationship with the biological behavior of human gastric carcinoma (14-24).

The suppression of Bax protein expression appears to contribute to gastric carcinogenesis. The expression of Bax protein in gastric precancerous lesions has recently been studied with immunohistochemistry indicating that Bax is expressed in gastric epithelial cells in all cases of chronic gastritis, but it was not detected in 26% of specimens of atrophic gastritis (10). As intestinal metaplasia and gastric dysplasia develop, Bax expression is further suppressed. Yang *et al.*. cocultured gastric adenocarcinoma SGC 7901 cells with a cytotoxic Helicobacter pylori strain, and showed that H. pylori induces apoptosis in gastric epithelial cells by upregulating Bax, and downregulating Bcl-2 proteins (12). Similarly, Konturek *et al.* showed that H.pylori infection in humans is associated with significantly upregulated expression of mRNA and protein for Bax and suppressed mRNA and protein expression for Bcl-2, as determined using RT-PCR and Western blot analysis (13).

Several investigators have shown that aspirin, and non-steroid anti-inflammatory drugs induce apoptosis in gastric cancer through upregulation of Bax (14-17). Recently, Tsunemitsu *et al.* used an adenoviral vector system expressing Bax protein in human gastric cell lines. Ad/Bax treatment was found to be effective in suppressing both subcutaneous and peritoneally disseminated MKN-45 tumours (18). Kim *et al.* indicated that the combination therapy of intratumoral administration of Bax gene complexed with a cationic lipopolyamine and chemotherapeutic agent, enhances the anti-tumoural effect of these agents (19).

Table 3. — **Univariate and Multivariate Analysis of Survival in studied Patients**

Variable		total	P^u	P^m	RR^m	CI^m
Age						
> =70	20	0.21	0.40	0.69	0.30-1.61	
< 70	24					
Sex						
Male	29	0.40	0.52	0.75	0.30-1.82	
Female	15					
Type						
Intestinal	22	0.72	0.71	1.16	0.51-2.61	
Diffuse	22					
Differentiation						
Poor	23	0.31	0.57	0.80	0.38-1.70	
Moderate	17					
Well	4					
Penetration through gastric wall						
T1-2	7	0.001	0.84	1.11	0.39-3.11	
T3	16					
T4	21					
Lymph node Involvement						
Positive	24	< 0.0001	0.31	0.41	0.07-2.29	
Negative	20					
TNM stage						
IB	6	< 0.0001	0.20	1.63	0.76-3.48	
II	10					
IIIA	9					
IIIB	3					
IV	16					
Bax expression						
Positive		20	< 0.0001	0.0487	3.34	1.0068-11.12
Negative	24					

P^u ; p value in Univariate analysis, P^m, RR^m, CI^m ; p value, Relative Risk, and Confindence Intervals in multivariate analysis, respectively.

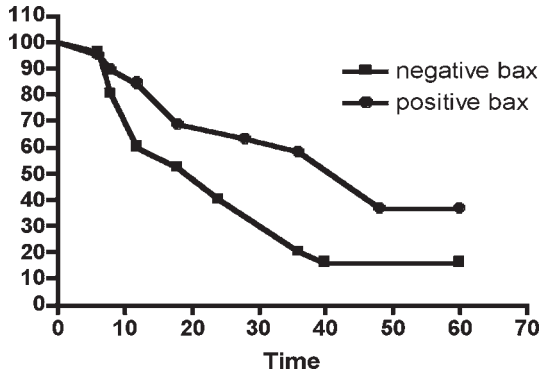

Fig. 2. — Kaplan-Meier survival analysis

Bax positive tumours have been shown to be more sensitive than Bax negative tumours to chemotherapeutic agents. Komatsu *et al.* investigated the effect of Bax overexpression in gastric cancer cell lines, using a Cre-loxP-mediated inducible expression system (20). After induction of Bax, cell lines showed decreased proliferation due to increased cell death, while Bax-expressing MKN-28 cells were more sensitive to cisplatin (20).

In this study, a significant association between Bax protein expression, differentiation degree of tumors and lymph node metastases was demonstrated. With regard to the histologic type of the tumor, Bax was less frequently expressed in intestinal than in diffuse type tumors. However, the difference was not statistically significant. These results are similar to those of Hai-Feng Liu *et al.* who studied the expression of Bax protein, with immunohistochemistry, in a series of gastric carcinomas (21). According to their results, the rate of Bax protein expression was strongly associated with the morphological type and differentiation degree of tumors. It was significantly higher in intestinal type and well differentiated gastric carcinoma than in diffuse type and poorly differentiated gastric carcinoma ($P < 0.05$ and $P < 0.01$).

Utilizing multivariate analysis, this study has shown that negative Bax expression is a negative prognostic factor for survival independent of sex, age, histological type of tumor, degree of differentiation, lymph node involvement and TNM stage. The median survival time of patients with negative and positive Bax protein expression was 48 and 24 months respectively ($p < 0.05$). Mrozek *et al.* also showed that the combined mutation of p53 and Bax in gastric cancer, two key regulators of the mitochondrial apoptosis pathway, results in an extremely aggressive tumor biology and poor clinical prognosis (22).

In conclusion, these findings indicate that a low Bax expression in gastric cancer is associated with de-differentiation, lymph node metastases, and poor clinical prognosis. Therefore, Bax protein expression may play an important role in the development and phenotypic differentiation of gastric carcinomas and tumor progression. Since experimental and clinical data also indicate that Bax protein may modulate the response to chemotherapeutic agents, the predictive value of Bax protein expression should be validated in large clinical trials utilizing different chemotherapeutic agents. Furthermore, Bax gene therapy has the potential to increase the antitumorous effect of chemotherapeutic agents.

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