Expression of apoptosis related genes bax, bcl-2 and bcl-X in human gastric cancer: early results of an investigation

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Abstract

Background. Evidences indicate an involvement of apoptosis related genes in gastric carcinogenesis. We studied the gene and protein expression patterns of *bcl-2*, *bax* and *bcl-X* in samples of gastric adenocarcinoma. The apoptotic index values, histological type, differentiation grade, cancer stage and lymph node status were statistically analysed for possible correlations with expressional data.

Methods. Thirty specimens of gastric cancer and respective normal control gastric mucosa were collected from patients with the diagnosis of gastric adenocarcinoma who underwent a curative gastrectomy. *bcl-2*, *bax* and *bcl-X_L* mRNA and protein levels were respectively determined by reverse transcription PCR (RT-PCR) and western blot using monoclonal antibodies for immunodetection.

Results and conclusions. We observed a significant suppression of *bax* with an increase of *bcl-X_L* at protein and mRNA levels. The presence of lymph node metastases was statistically related to the loss of *bax* overexpression. *Bcl-X_L* was mostly up-regulated in intestinal/mixed types of gastric carcinoma. The expression patterns described confirm the role for these apoptosis genes in gastric adenocarcinoma.

Key words: gastric cancer, apoptosis, bax, bcl-2, bcl-X

Introduction

Gastric cancer is the second-leading cause of cancer related deaths worldwide, approximately 15,000 deaths per year, with the highest frequency rates registered in Japan (78/100,000) and Eastern Asia countries.[1,2] Its incidence has recently declined in many countries, probably due to significant modifications in life-style, eating habits and exposure to environmental factors.[3]

The development of gastric cancer is considered a multistep process in which environmental carcinogens and individual genetic factors determine the transformation of normal gastric epithelium into neoplastic cells. The environment plays a major role, as confirmed by migration studies, showing a decrease in gastric cancer incidence when populations moved from high-risk to low risk regions.[4] Epidemiology indicates high calorie diet, red meat or salty foods uptake and *Helicobacter pylori* infections as main risk factors.[5] Recent studies have enlighted the role of apoptotic genes, showing how a disturbed balance between cell survival and programmed cell death is closely related to gastric cancer development.[6] The bcl-2 family of proteins is one of the most studied. It comprises of both pro- and anti-apoptotic regulators of programmed cell death, including the *bcl-2* protooncogene, coding for an anti-apoptotic protein; the *bax* gene, an antagonist of bcl-2 and thus promoter of apoptotic cell death; *bcl-X*, coding for two proteins, bcl-X_L and bcl-X_S, with distinct apoptotic functions, the former inhibiting apoptosis as effectively as the bcl-2 protein, the latter blocking the death suppressor activity of bcl-2.[7] There is evidence that tumour development is associated with the inactivation of *bax* and with the overexpression of *bcl-2*, leading to inhibition of apoptosis.[8] Konturek et al. observed a significant up-regulation of the *bcl-2* gene, together with a down regulation of the pro-apoptotic *bax* gene in gastric cancer.[6] In a study based on both reverse transcription PCR (RT-PCR) and immunohistochemistry, Kondo et al. observed a consistent overexpression of *bcl-X_L* and *bcl-2* genes in over 67% of intestinal type of carcinomas, and in 40% of diffuse type of carcinomas according to the Lauren classification.[9,10]

Apoptosis dysregulation mechanisms seem to play an important role in the early steps of gastric tumourigenesis, but further epidemiological and experimental studies are required to evaluate the effective involvement of apoptotic pathways. [9,11,12]

In this paper we studied the expression of *bcl-2*, *bax* and *bcl-X_L* apoptotic genes both at the mRNA and the protein level in order to identify reciprocal expression patterns and to verify the possible associations with clinical parameters.

Methods

Patients

Our series includes 30 patients - 16 males (53.3%) - of an age ranging from 39 to 88 years (mean value: 64, sd ±12.4), with a diagnosis of gastric adenocarcinoma, who underwent a curative gastrectomy between November 1999 and May 2000 at "A. Gemelli" General Hospital of the Catholic University, Rome. Patients were enrolled after submission of giving informed consent. Primary tumour location was the lesser curvature and/or gastric body in 9 cases (30.0%), the gastric antrum in 16 (53.3%), the cardias in 4 (13.3%) and the gastric stump in 1 (3.3%). Twenty-three patients were submitted to a subtotal gastrectomy with tumour-free margins (76.7%) and 7 to a total gastrectomy (23.3%); in all of the patients of this series we accomplished a so-called D2 lymph adenectomy, with a total of 1343 nodes removed nodes and nodes with an average of 44.8 nodes per case (range: 18 – 92 nodes). 360 (26.8%) of all of the nodes removed nodes were metastatic at pathological examination, with an average of 12 positive nodes per patient. 80% (24/30) of patients had a lymph node involvement.

Specimen collection

Fresh samples of gastric carcinoma and normal control gastric mucosa adjacent to neoplastic tissue (≥0.5 cm, remaining in the same gastric region, with no evidence of dysplasia) were collected, stored in liquid nitrogen and submitted to histopathologic, molecular and immunohistochemistry analyses.

Histopathologic analysis

Patients were staged according to the latest TNM classification.[13] The evaluation of histologic type was performed in accordance with the Lauren classification.[10] According to tumour grading, patients were classified as follows: well-differentiated (G1), moderately differentiated (G2) or scarcely differentiated (G3).

Molecular analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate method.[14] Both control mucosa and tumour samples were carried out in parallel under identical conditions. First-strand cDNA was prepared in a 20 µl final volume by adding 1 µg of total RNA to the following mixture: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 20 mM dNTP, 20 pmol random hexamers, 20 U RNase Inhibitor (Amresco), 5 mM DTT and 200 U MMLV-Reverse Transcriptase (MMLV-RT, Gibco BRL). Tubes were incubated in a Thermal Cycler (Eppendorf, Mastercylcer Gradient) for 5 minutes at 65°C, for 65 minutes at 37°C and 5 minutes at 95°C. 2 µl of cDNA were amplified by using 1 U Taq DNA polymerase (Boehringer Mannheim) with 15 pmol each primers, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 300 mM dNTP in a 30 µl final volume.[15] Cycling was performed for 25 cycles according to the following parameters: denaturation at 94°C for 1 min, annealing at 60°C for 45 seconds, extension at 72°C for 2 minutes. To allow semi-quantitative analysis, β-actin was co-amplified as an internal control. 12 µl of each product were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV fluorescence; images were scanned and bands were compared using Quantity One software (Biorad). For the determination of *bcl-2, bax* and *bcl-X_L* mRNA levels, the peak-height ratio of the signals obtained by RT-PCR to β-actin fragment were measured. The results were expressed as equal, decreased (<1.5 fold) or increased (>1.5 fold) expression of the gene in the tumour tissue compared to normal mucosa.

Immunohistochemistry analysis and scoring method

For the immunohistochemistry analysis, the specimens were fixed in 4% buffered formalin and paraffin embedded. Sections were stained with hematoxylin and eosin for histological evaluation. Sections were incubated with anti bcl-2 (YLEM) mouse monoclonal antibodies, and bax and bcl-X (the latter detecting both L and S proteins) (Santa CruzBiotech) rabbit polyclonal antibodies; following incubation with avidin-biotin-peroxidase (ABC method) (Vector, Burlingame), the reaction was detected with 3.3'-diaminobenzidine (Vector). Negative controls were performed using istotype specific and rabbit pre-immune sera for mouse monoclonal and rabbit polyclonal antibodies, respectively. The results were scored considering, in for each sample, both the percentage of immunoreactive tumour cells and the prevailing intensity of immunostaining, according to Koshida et al. [12]

In brief, the former was classified as follows: 1 - less than 5%; 2 - from 6 to 20%; 3 - from 21 to 50% and 4 - over 50%; the latter was divided into five groups: 0 - completely negative; 1 - very weak; 2 - weak; 3 - moderate and 4 - intense (the value found in the majority of the immunoreactive cells was adopted).The final score of immunoreactivity for each sample was calculated by multiplying the values of the two parameters. For all of the proteins investigated, the results for immunoreactivity was also expressed for each sample as equal, decreased or increased after comparing the immunohistochemical final scores of tumour tissue versus the control non-tumoural whole mucosa or foveolar mucosa, the latter being the putative site of origin of the gastric carcinoma.[16] Lymphocytes, activated lymphocytes and plasma cells, and as well as small vessels were used as internal positive controls for bcl-2, bax and bcl-X immunoreactivity, respectively.

Apoptotic index

Apoptosis was investigated using the TUNEL procedure technique and the results were expressed as the percentage of apoptotic cells with respect to the total number of the neoplastic ones.[17]

Statistical Analysis

For each patient the following data were collected: age, gender, tumour size, lymph node metastasis, disease stage, tumour grading, values of apoptotic index of tumour tissue, and values of apoptotic index for normal mucous tissue. *Bcl-2, bax* and *bcl-X_L* genes expression at mRNA level were classified as normal, increased or decreased on the basis of the established criteria. Histochemical immuno-staining intensities of bax, bcl-2 and bcl-X proteins were evaluated as continuous variables. Median and range were selected respectively as the measures of central tendency and variability due to the skewed distribution of variables being investigated. The association between *bax, bcl-2 and bcl-X* expression and various clinicopathological features of gastric carcinoma was analysed using Chi-square or Fisher's exact tests when appropriate. Friedman test for K related samples was used to correlate the final score of immunoreactivity for bax, bcl-2 and bcl-X proteins in tumour tissue, control whole mucosa and non-tumoural foveolar mucosa. A p value of less than 0.05 was considered statistically significant. All analyses were carried out using STATA version 7.0 (STATA Corp, College Station, TX).

Results

Histopathologic analysis

Based on the histo-pathological reports, patients were staged as follows: 16.7% stage I (5 cases), 23.3% stage II (7 cases), 30.0% stage III (9 cases) and 30.0% stage IV (9 cases); with regard to T and N status, 3 patients had a pT1 tumour (10.0%), 13 pT2 (43.3%), 14 pT3 (46.7%); 6 patients were pN0 (20.0%), 9 pN1 (30.0%), 6 pN2 (20.0%) and 9 pN3 (30.0%). According to Lauren classification, gastric carcinomas were divided into intestinal and diffuse histologic types. Intestinal adenocarcinoma was the most commonly observed histologic type (15 patients, 50.0%, including one case with neuro-endocrine features and one of the mucinous subtype), diffuse carcinoma was observed in 13 patients (43.3%) and "mixed" carcinoma in 2 (6.7%). According to tumour grading, patients in this series were classified as follows: well-differentiated (G1) 1 case (3.3%); moderately differentiated (G2) 4 cases (13.3%); and scarcely differentiated (G3) 25 cases (83.3%).All the cases of diffuse type of carcinoma were considered G3 (scarcely differentiated).

Molecular analysis

Bcl-2 family genes showed differences in mRNA expression between gastric cancer and mucosa samples; mRNA expression were detectable in 24 (80%) out of 30 samples. *Bcl-X_L* expression was increased (1.5-12.7 folds) in 45.8% (11/24) of cases, equal in 33.3% (8/24) and decreased (1.5-2.8 folds) in 20.8% (5/24) of samples. *Bcl-2* gene expression was identical in 41.67% (10/24) of tumour samples, increased (1.5-9.4 folds) in 20.8% (5/24) and decreased (1.5-10 folds) in 37.5% (9/24) of samples. *Bax* mRNA was increased (1.5-8.6 folds) in 41.67% (10/24) of tumour samples, decreased (1.5-30 folds) in 8.3% (2/24) of samples, while in 50% (12/24) of cases we observed a slight decrease or no significant differences in tumour versus paired mucosa.

Immunohistochemistry analysis and scoring method

The final score of immunoreactivity for bax, bcl-2 and bcl-X proteins in each sample was independently calculated in the tumour tissue samples, as well as for the control whole and foveolar mucosa; the results are shown in Table 1. Bax immunoreactivity was completely negative in 43% (13/30) of tumour samples, while bcl-X protein was detectable in 100% of gastric cancer samples, with an high immunoreactivity score (value $\geq$12) in 56.6% of the samples; immunopositivity (value $\geq$1) for bcl-2 was found in

Table 1. Median value (range when more than one value available) of final score of immunoreactivity for bax, bcl-X and bcl-2 proteins in tumour tissue, non-tumoural whole-thickness mucosa and non-tumoural foveolar mucosa.

	bax Median (range)	p*	bcl-X Median (range)	p*	bcl-2 Median (range)	p*
Tumour tissue	2 (0-12)		12 (2-16)		-	
Non-tumoural whole thickness mucosa	4 (4-12)	<0.001	8 (4-12)	<0.001	3 (0-9)	<0.001
Non-tumoural foveolar mucosa	4		8 (4-8)		-	

* Friedman test for K

8 out of 30 tumour samples (26.6%). The immunoreactivity scores for bax, bcl-2 and bcl-X proteins were compared in tumour samples versus paired non-tumoural whole-thickness mucosa tissues, showing a decrease in bax reactivity in 70% (21/30) of cases, and an increase in bcl-X reactivity in 66.67% (20/30) of cases, as reported in Table 2. The resultant bcl-2 immunoreactivity was reduced in the tumour tissues as compared to the whole mucosa in 80% (24/30) of cases. The same comparison was made for tumour tissue versus foveolar mucosa. These results showed a similar trend, but the following major differences: a decrease in bax reactivity in 40% (12/30) of cases, and an identical immunoreactivity score for bcl-2 in 66.6% (20/30) of cases (Table 2).

Apoptotic index

The median value of apoptotic index was calculated both for gastric cancer tissue and mucosa samples and the results were 2.75 (range 0.5-7.5) and 0.5 (range 0.13-3.5), respectively (p<0.001).

Statistical analysis allowed to considered the possible correlations between apoptotic genes expression, tissue immunoreactivity, apoptotic index and several clinicopathological features. A significant correlation was observed between the *bax* gene expression at mRNA level and lymph node metastases (p=0.006), as reported in Table 3. When *bax* expression was found to be equal or slightly decreased, all of the patients had lymph node metastases. An inverse correlation was also found between *bax* expression and the pathological staging of for cancer (p=0.05); the 78.5% (11/14) of patients with a normal or lowered tumoural *bax* expression had an advanced (III-IV) stage of cancer. Non-statistically significant correlations were found between *bcl-2* oncogene expression and the clinicopathological features of the studied cases; however, 80% (4/5) of cases with increased *bcl-2* mRNA were early stages (I-II) cancer. We found a significant correlation between *bcl-X_L* oncogene expression and the histological type of the gastric cancer: between tumours hyperexpressing *bcl-X_L*, 81.8% (9/11) were intestinal/mixed type carcinomas (p=0.027; Table 3). No significant correlation was found between *bcl-X_L* expression and the stage of cancer: among *bcl-X_L* hyperexpressing tumours, 54.5% (6/11) were in an advanced stage. We could not observe significant statistical correlation between the protein immunoreactivity rate of tumour with respect to non-tumoural mucosa and the clinicopathological features considered. The median value of apoptotic indexes of early stage (I/II) or advanced stage (III/IV) and differentiated (G1/G2) or undifferentiated cancers was 3.7 (range: 0.5-7.5) and 2.3 (range: 0.5-6.5), 4.5 (range: 2.5-7.0) and 2.6 (range: 0.5-7.5), respectively. Apoptotic index did correlate with protein expression, given that when bcl-X and bcl-2

Table 2. Comparison of bax, bcl-X and bcl-2 immunoreactivity levels between tumour tissues and non-tumoural whole-thickness mucosa and non-tumoural foveolar mucosa.

	bax tvsm* N (%)	bcl-X tvsm N (%)	bcl-2 tvsm N (%)	bax tvsf** N (%)	bcl-X tvsf N (%)	bcl-2 tvsf N (%)
Equal	6 (20)	5 (16.67)	6 (20)	13 (43.33)	4 (13.33)	20 (66.67)
Increased	3 (10)	20 (66.67)	0	5 (16.67)	22 (73.33)	8 (26.67)
Decreased	21 (70)	5 (16.67)	24 (30)	12 (40)	4 (13.33)	2 (6.67)
Total	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)

*tvsm: tumour compared to non-tumoural whole-thickness mucosa

**tvsf: tumour compared to non-tumoural foveolar mucosa

Table 3. Relationship between apoptotic genes expression in tumour tissues compared to non-tumoural whole-thickness mucosa samples and clinicopathological features.

	bax			$bcl\text{-}X_L$				bcl-2			
	Increased (n=10)	Equal/Decreased (n=14)	p*	Increased (n=11)	Equal (n=8)	Decreased (n=5)	p*	Increased (n=5)	Equal (n=10)	Decreased (n=9)	p*
Lymph node metastases N(%)	5 (50)	14 (100)	0.006	9 (81.8)	7 (87.5)	3 (60)	ns	3 (60)	9 (90)	7 (77.7)	ns
Staging >II N(%)	4 (40)	11 (78.5)	0.054	6 (54.5)	6 (75)	3 (60)	ns	1 (20)	8 (80)	6 (66.6)	ns
Grading >I N (%)	7 (70)	13 (92.8)	ns	7 (63.6)	8 (100)	5 (100)	ns	4 (80)	5 (50)	9 (100)	ns
Intestinal type N (%)	6 (60)	7 (50)	ns	8 (81.8)	2 (25)	3 (80)	0.027	3 (60)	7 (80)	3 (44.4)	ns

* Chi-square test

immunoreactivity were down regulated, the resultant apoptotic index resulted increased (median values: 4.1 ±2.27 and 3.4 ±1.9, respectively). We could observe a similar correlation for the proapoptotic *bax* oncogene, given that the apoptotic index was increased (median value: 4.45 ±0.55) when the gene was overexpressed.

Discussion

Apoptosis is an active process with a fundamental role for in tissue development and differentiation. In the gastrointestinal tract, a balance between cell growth and apoptosis influences mucosa homeostasis, so that its dysfunctions may determine cell transformation and cancer development.[18, 19] Programmed cell death is a complex phenomenon where the bcl-2 family of proteins seems to play a key role. In the present study, we investigated expression of *bcl-2*, *bax*, *bcl-X* apoptotic genes in gastric cancer with respect to the corresponding non-neoplastic gastric mucosa. The analysis was performed both at transcription and translation level by means of RT-PCR and immunohistochemistry. Expression data where considered for possible statistical associations with clinicopathological parameters.

We observed a significant suppression of bax protein expression in cancer tissues (70% of cases), and bax immunopositivity (value ≥1) was found in only 57% of the gastric cancer tissue. *Bax* gene product is supposed to act as an accelerator of apoptosis, opposing *bcl-2* effects on cell-cycle.[20] Previous studies *in vitro* showed that the overexpression of *bax* in gastric cell lines decreases tumour cell growth due to induction of programmed cell death.[21] A down regulation of *bax* oncogene could be related to decreased apoptosis and accumulation of neoplastic cells, even if *in vivo* the complex interaction of different genes does not support the specific and self-sufficient role for a single gene. In contrast to *bax*, the gene expression of $bcl\text{-}X_L$ appears to be increased at the mRNA level (45.8% of cases) and at the protein level (66.7% of cases), in tumours compared to the whole normal gastric mucosa.An overexpression of $bcl\text{-}X_L$ in gastric carcinoma was already observed by Kondo et al. [9] which suggested that a dysregulation of apoptosis by an overexpression of this gene could play a role in gastric carcinogenesis. Bcl-2 protein was detected (immunoreactivity score value ≥1) only in 26.6% of the gastric cancer samples. This observation remains in agreement with a previous study by Saegusa et al. who demonstrated bcl-2 immunopositivity in 14% and 12.6% of gastric cancer samples.[11, 22]. Similar expression levels of *bcl-2* oncogene were detected in the majority of the tumour tissue samples compared to the normal mucosa, as also confirmed also by immunohistochemistry. An overexpression of bcl-2 gene was present in only in 20.8% of tumour samples, and a similar trend (26.6% of increase) was found to be at the protein level when foveolar mucosa was considered as the control.

The correlation of the apoptotic oncogene expression with clinicopathological parameters demonstrated a statistically significant relationship between *bax* expression and the presence of lymph node metastases and between *bax* expression and cancer stage. Only a minority of patients with lymph node metastases and with an advanced stage of cancer overexpressed *bax* oncogene. These new data suggest that *bax* gene could play a role in cancer progression especially in the later stages.The loss of its overexpression may be a step in the loss of an appropriate pro-apoptotic effect in the cell hyperproliferation, particularly when antiapoptotic genes like $bcl\text{-}X_L$ are overexpressed too. Also noteworthy is the statistically significant higher expression of $bcl\text{-}X_L$ oncogene in the intestinal/mixed type of carcinoma with respect to the diffuse type, as already described by Kondo et al. and Krajewska et al.[9,23] In accordance with previous reports, statistical analysis of clinicopathological features did not show significant correlation with regard to oncogene data obtained by immunohistochemistry.[23] These

results could be biased by the limited size of the population sample and more specific biomedical parameters could be considered in further studies, as well as more advanced and sensitive techniques, such as real time PCR or microarray analysis.[24].

We observed an apoptotic index higher in the early stage lesions (median value: 3.7) than in the advanced ones (median value: 2.3), in contrast with previous reports.[12] This may be due to differences in patient sampling, drug treatments or other confounders. Our findings show a statistically significant *bax* slight down regulation in advanced stages as well as bax protein down regulation mostly in advanced stages (Table 3). Moreover, even if *bcl-2* gene overexpression was mostly observed in the early stages of cancer, we also could not detect it also at protein level, probably due to post-transcriptional events and lack of protein synthesis. *Bcl-X_L* gene expression was not associated with the stage of cancer, its increase being mostly identically distributed in early and advanced stages of cancer. When normal mucosal foveolae were taken as control, instead of whole-thickness mucosa, immunohistochemistry revealed a substantial similarity in bcl-2 staining (66.67% of cases; Table 2). Actually, in only 10 cases it was observed a variation in immunoreactivity levels and 80% (8/10) of these showed an increase of bcl-2 immunoreactivity. Since foveolae are the putative site of origin of for gastric carcinoma this aspect may have a consistent biological value and it could might be advisable to address direct future studies towards a more specific comparison of oncogene levels between cancer tissue and foveolar mucosa.[22] Combination of both transcriptional and protein-level data did not improve statistical analysis neither or produceed any further significant correlation with clinicopathological parameters (data not shown).

In conclusion, our data confirm that a dysregulation of apoptosis occurs in the development of gastric cancer and that a hypoproduction of bax protein together with an up-regulation of *bcl-X_L* could play a determinant role in this event. We wanted to compare expression modification at mRNA transcription level with gene product synthesis. The reported observations support the presence of modifications at post-translational level and reduce the role of single apoptotic gene transcription as a sensible and specific marker associable with clinical or pathological features. A limitation which may have biased our results is represented from the high proportion of patients who were already in a late stadium of the disease (9/30 stage IV). Presently, this and other studies are based on basic molecular methods and limited population sample sizes, but report interesting oncogene expression patterns supporting the involvement of apoptosis in gastric cancer. Rapid technical advances in molecular biology are opening up new perspectives for understanding the role of apoptotic genes in carcinoma progression.

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