RESEARCH COMMUNICATION

Apoptosis Dysregulation in Human Gastric Carcinomas: Relationship to Anti- and Pro-Apoptotic Protein Expression

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Abstract

Apoptosis and the genes regulating this process have recently become a focus of interest in the study of cancer development and progression. Both Bcl-2 and Bax are transcriptional targets for the tumor supressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage. The coordinate performance of these molecules is crucial for controlling life or death of a cell. Correlations between apoptosis and protein expression of genes controlling this process including Bcl-2, Bax and p53 in gastric cancer were here investigated with gastric tumor samples of forty patients . DNA ploidy pattern was anlyzed using flow cytometry and Bcl-2, Bax, and p53 were immunohistochemically localized using specific monoclonal antibodies. In addition, serum Bcl-2 protein was estimated by enzyme linked immunosorbant assay (ELISA). The obtained data showed that the mean serum Bcl-2 protein concentration demonstrated a significant increase (P<0.0001) in positive cases (61.5±11.0 unit/ml) compared to the negative ones (47.5±3.5 unit/ml). Serum Bcl-2 protein positivity was detected in 13/40 of gastric cancer patients. Immunohistochemical positivity for Bcl-2, Bax, and p53 was shown in 45%, 68%, and 63% of samples, respectively. Positive Bcl-2 and p53 immunostaining was significantly linked with the histological grade (P<0.02 and P<0.009 respectively) and lymph duct invasion (P<0.02 and P<0.001). On the other hand, Bax was significantly differed with lymph duct invasion and the ploidy pattern (P<0.03 and P<0.002). In conclusion, the apoptosis-related genes p53, Bcl-2, and Bax are all linked to the occurrence of gastric cancer. Therefore, analysis of their expressions may add useful information concerning tumor behavior.

Key Words: Apoptosis - Bax - Bcl-2 - p53 - gastric cancer.

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Introduction

Apoptosis (programmed cell death) is a key mechanism causing cell death and organ diseases, failure of apoptosis is now understood to contribute to the development of human malignancies (Deng et al, 1999; Zhao, 2000)). Apoptosis and the genes regulating this process have recently become a focus of interest in the study of cancer development and progression (Eissa and Seada, 1998). Several genes seem to be involved in the induction as well as the inhibition of apoptosis, these genes are as important as the oncogenes that exert their effect by accelerating of cell proliferation (Kandouz et al, 1996; Ye et al, 2005).

The Bcl-2 family of proteins includes the best characterized regulators of apoptosis, comprising anti-apoptotic members, and pro-apoptotic members (Nomura et al, 2003). Many of the pro-apoptotic family members, such as Bax, Bid, Bad, Bim, and Bmf, are localized in the cytoplasm, and apoptotic stimulation results in their translation to the mitochondria and induction of the release of apoptogenic factors, probably by inactivating anti-apoptotic members of the family and activating multi-domain members like Bax and Bak (Puthalakath and Strasser, 2002). Proteins of this family regulates a distal step in an evolutionarily conserved pathway for programmed cell death (Reed, 1994). Despite intensive investigation, the mechanisms by which Bcl-2 and its homologues control life and death largely remain enigmatic, but interactions between these Bcl-2 family proteins through the formation of Homo-and hetero-typic dimmers and the ratio of anti- to proapoptotic members appear to be crucial (Lan and Lu, 2004).

One of the most intensly studied cancer - related genes is the p53 tumor suppressor gene (Carson and Lois 1995). Wild type p53 modulates cell proliferation and differentiation by regulating the transcription of several gene products, inducing p21waf1 expression which acts as a regulator of the cell cycle at the G1 checkpoint, and plays a crucial role in repairing damaged DNA through inducing GADD45 (growth arrest and DNA damage) (Zhan et al, 1994). Moreover p53 induces the Bax gene, which is followed by apoptosis, in contrast to Bcl-2 (Lan et al, 2003).

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However, delineating those genes and correlating molecular events with clinicopathological characteristics may lead to important new insights into the pathogenesis of gastric cancer (Tahara, 1995). Therefore, this study was conducted to evaluate correlations between apoptosis and related gene products (Bcl-2, Bax, and p53) with clinico-pathological parameters of patients with gastric cancer.

Patients and Methods

Patients and tissue specimens

The present study was conducted on 40 gastric cancer patients with age range from 20 to 76 years (mean 50.65) including 24 men and 16 women (M/F 3:2). Blood samples and tissue specimens were collected in the Gastroenterology Surgical Center, Mansoura University, Mansoura, Egypt. Tissue specimens were divided into two parts; one for processing for flow cytometry analysis and the other part fixed in formalin for immunohistochemical examination. Hematoxylin and eosin stained sections were histopathologically assessed for classification according to the histological type and tumor staging. Informed consent was obtained from all patients, and the local ethical committee at the Gastroenterology Surgical Center, Mansoura University, Mansoura, Egypt, approved the study protocol.

Immunohistochemistry of Bcl-2,Bax and P53

Bcl-2, Bax, and p53 were analyzed immuno-histochemically using the streptavidin-biotin peroxidase method with monoclonal antibodies specific for Bcl-2, Bax, and p53 (Dako,Copenhagen, Denmark). 4-5 μm-thick sections were rehydrated in descending grades of ethyl alcohol. Antigen retrieval (AR) was performed by boiling in 10mM/L citrate buffer (pH 6) for 30 minutes, then the sections were left to cool to room temperature and washed with PBS pH7. After the endogenous peroxidase was quenched by 3% hydrogen peroxide/ methanol for 5 minutes, the sections were incubated with primary mouse monoclonal antibody (p53 at a dilution 1:50 overnight, Bcl-2 1:40 and Bax at a dilution 1:50 for for one hour at room temperature). Biotinylated secondary antibody, rabbit antimouse were added with subsequent addition of avidin-biotin peroxidase complex. The color was developed by using 3-amino-9-ethyl carbazol (AEC) as the chromogen and the sections were counter stained with Mayer's Hematoxylin. Positivity was detected guided by positive control (sample known to be positive for the antigen tested) and negative control for each sample (the primary antibody was omitted).

Apoptotic index

Apoptosis was detected in H&E stained sections and expressed as an apoptotic index (AI), defined as the percentage of tumor cells positive for apoptosis in 100 tumor cells from three arbitrarily selected microscopic fields.

Quantitative measurement of Bcl-2 protein by Enzyme-Linked Immunosrbent Assay (ELISA)

Serum concentrations of the Bcl-2 protein were analyzed by Bcl-2 ELISA kit provided by oncogene research products (Oncogene Science, Cambridge, UK). The Bcl-2 ELISA is a "sandwich" enzyme immunoassay employing mouse monoclonal antibodies. An antibody, specific for the human Bcl-2 protein, has been immobilized onto the surface of the plastic wells provided in the kit. The sample to be assayed and FITC conjugated detector monoclonal antibody are pipetted into the wells and allowed to incubate for two hours, during which any Bcl-2 binds to the capture and detecting antibodies. Unbound material is washed away, and horseradish peroxidase-conjugated anti-FITC antibody is added,which binds to the detector antibody. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent), the intensity of which is proportional to the amount of Bcl-2 protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentration of Bcl-2 (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of Bcl-2 with that obtained from the standards, the concentration of Bcl-2 in the sample can be determined. Cutoff level of ELISA above or below which the tested samples is considered positive or negative was calculated as the mean concentrations of 20 serum samples from healthy individuals +2SD.

Flow cytometry analysis

DNA ploidy analysis of DNA content of fresh gastric cancer specimens was carried out as described by Attallah et al, (1997). Analysis was carried out using flow cytometry (Coulter EPICS profil II, Coulter Corp., Hialeah, F1, USA). The cellular amount of DNA was expressed as DNA index (DI). Cells with a normal diploid DNA fluorescence distribution were identified relative to human peripheral blood as external DNA reference standard. Following identification of the diploid G0/G1 peak, DI value of 1.0 indicates diploid. A tumor sample was defined as aneuploid when bimodally separated population of G1 cells was present and was calculated as the ratio of the aneuploid G1 peak to the diploid GI peak in the same sample.

Statistical analysis

Results were expressed as mean or mean ±SD if applicable and were analyzed by using the $\chi 2$ test, Student's T test, Fisher's exact test as appropriate. The Student's T test was used to compare different groups for continuous variables including the serum levels of Bcl-2. Differences in positivity of Bcl-2, Bax, and P53 according to characteristics of patients were compared with the Chi-square test or Fisher's exact test. SPSS 11 for Windows statistical software package (SPSS Inc., USA). was used for all the statistical analyses. A P value < 0.05 was considered statistically significant.

Results

Apoptosis was observed in 90% (36/40) of cases with

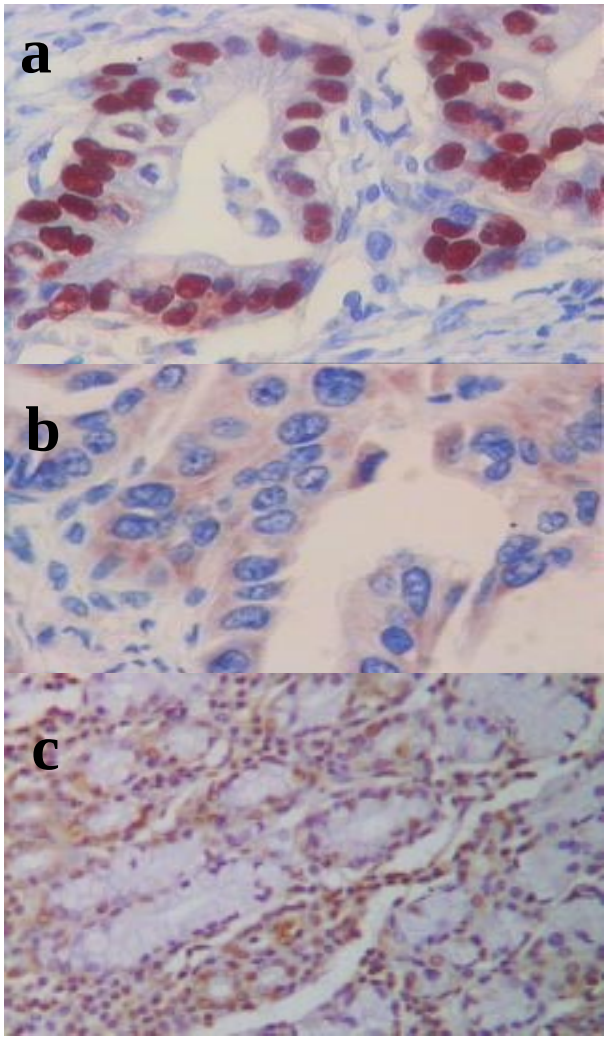

Figure 1. Immunohistochemical staining of p53 (a), Bcl-2 (b), and Bax (c) . Counter staining with Mayer's hematoxylin

gastric cancer. The positivity for apoptosis was recorded in 87.5%(7/8) of grade I, in 90 %(18/20) of grade II, and in 91.7%(11/12) of grade III.

Bax immunopositivity was found in 68 % (27/40) of cases with gastric cancer (Fig.1). The positivity was increased with the progression of gastric cancer, where the incidence of Bax immunostained cases was detected in 50% (4/8) of grade I, 70% (14/20) of grade II, and in 75% (9/12) of grade III (Fig. 2). There is no significance of Bax expression with the pathological parameters including histological grade, tumor size, gross picture, tumor type, depth of invasion, stromal reaction, lymph node metastasis and tumor site (P>0.05). On the other hand, Bax immunoreactivity was satistically significant with lymph duct invasion (P<0.03) and polidy pattern (P<0.002).

The concentration of Bcl-2 protein was measured by ELISA and the positivity was detected in 32.5% (13/40) of cases. Mean serum concentration of Bcl-2 in the positive cases was 61.47±10.99 unit/ml demonstrating a significant elevation (P<0.0001) compared to the negative cases (47.55 ± 3.5 unit/ml) where the mean concerntartion of Bcl-2 in the serum of normal controls (44.9± 4.2 unit/ml) and the cut off value (53.4 units/ml).

Bcl-2 protein was immunohistochemically localized in the cytoplasm of 45% (18/40) of gastric cancer patients (Fig. 1). The incidence of Bcl-2 immunostained cases was detected in 87.5% (7/8) of grade I, 35% (7/20) of grade II, and in 33.3% (4/12) of grade III (P<0.02) (Fig. 2). Mean Bcl-2 concentrations were 54.13±13.52 in grade I; 49.2±4.7 in grade II; and 55.5±11.36 in grade III; showing a significant difference between grade II and III only (P<0.03). However, no significance was found between Bcl-2 expression by immunostaining and tumor size, gross, type, depth of invasion, stromal reaction, and L.N metastasis. A significant difference was found between Bcl-2 expression and lymph duct invasion only (P<0.02).

The expression of p53 was detected by immunohistochemistry in 63% (25/40) of gastric cancer patients. Nuclear immunoreactivity was detected as diffuse pattern in 52% (13/25) and focal pattern in 48% (12/25) of p53 positive cases (Fig. 1). The positivity for p53 was increased with the progression of gastric cancer (Fig. 2) with siginificant difference with the histological grade (P<0.009), and lymph duct invasion (P<0.001). No significance of p53 expression with the tumor size, gross picture, tumor site, lymph node metastasis, and ploidy pattern. However, an inverse expression was detected for p53, Bcl-2, and Bax in respect to the histological grade (Fig. 2) but there is no significant correlation between them.

Cases positive for apoptosis showed variable expression of Bcl-2, Bax, and p53 (Fig. 3). Expression of bcl- 2 was increased stepwise from mild to moderate apoptotic index (AI) but negative expression was observed

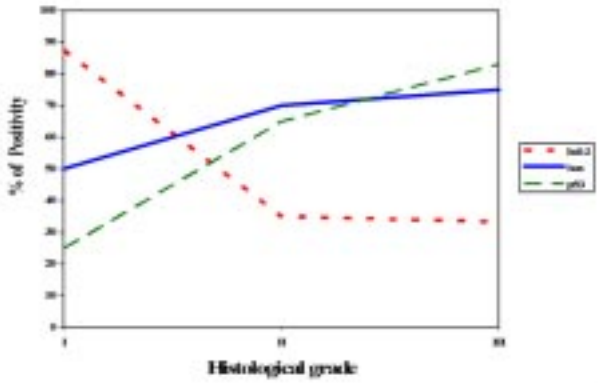

Figure 2. Bax, Bcl-2, and p53 Protein Expression in Gastric Cancers of Different Grades

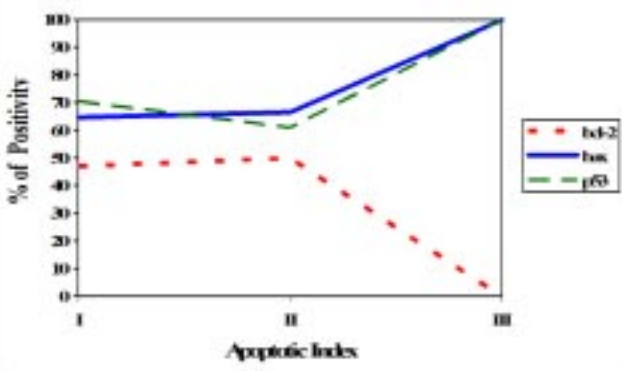

Figure 3. Relationship between Bcl-2, Bax, and p53 Protein Expression and Apoptotic Indices

at severe AI. On the other hand, Bax and p53 expression were relativly stationary with mild and moderate AI. However, higher expression was observed with increasing of AI. Howvere, there is no significance between AI with clinicopathological variables or DNA ploidy pattern but a significant correlation was observed between AI and L.N metastasis (P<0.04).

Discussion

Altered apoptosis contributes to a number of human diseases, including cancer, autoimmune diseases, and neurodegenerative disorders (Thompson, 1995; Bani-Hani et al, 2005). Apoptosis plays a significant role in human gastric carcinogenesis (Ishida et al, 1996; Liu et al, 1998;Liu et al, 1999).

Apoptosis was detected in the present study in 90% (36/ 40) of cases with gastric cancer; the positivity was recorded in 87.5% (7/8) of grade I, in 90% (18/20) of grade II, and in 91.7% (11/12) of grade III. Hino et al, (1996) reported that, the incidence of apoptosis increased as the differentiation grade of hepatocellular carcinoma was lowered, suggested a rapid turnover of cancer cells in the lower differentiation grades. Kasagi et al, (1994) studied the apoptosis indexes at various levels of differentiation and found some difference between tumor tissue of high and low differentiation. The difference indicated that gastric cancer with a low degree of differentiation was less likely to undergo apoptosis.

The apoptosis related genes are expressed in various frequencies in different cancers, and a general pattern of activation or inactivation of these genes in malignant tumors can not be defined. Therefore, the function of apoptosis genes in different human cancer must be evaluated individually (Guo et al, 2002). The Bcl-2 related genes regulate cell death and are considered to correlate with the pathogenesis and progression of cancers (Chen et al, 2000;Yang et al, 2001). The most extensively studied anti-apoptotic gene is Bcl-2 (Craig, 1995). The Bcl-2 protein has been shown to block or delay apoptosis. The Bcl-2 gene encodes an intracellular protein associated with mitochondrial and nuclear membranes and with the endoplasmic reticulum (Hockenbery et al, 1990).

In the present investigation, immunoreactivity of Bcl-2 was detected in 45% (18/40) of gastric cancer patients. The positivity for Bcl-2 was detected in 87.5%, 35%, and 33.3% in grade I, II, III respectively (P<0.02). These results denoting that the expression of Bcl-2 decreased with the progression of gastric cancer as reported by Xu et al, (2001), and the expression of Bcl-2 reach the top at the early stage of gastric cancer and decreased in the advanced lesions of gastric cancer. Bcl-2 protein was detected by ELISA assay in the sera of 32.5% (13/40) of our patients. Mean serum concentration of Bcl-2 positive was elevated significantly (P<0.0001) in positive cases compared to negative ones. The expression of Bcl-2 was reported to increase in cells of gastric cancer (Sundblad and Tamayo, 1995). The expression of Bcl-2 is a phenomenon that occurs in the early period in the development of gastric cancer. Therefore, Bcl-2 might do some work both in the triggering of gastric cancer and developing of early gastric cancer (Saegusa et al, 1995).

Although Bcl-2 was a strong inhibitor to apoptosis, it could not induce the cancer alone (Xu et al, 2001). Bax is a protein that constitutes an isomeric dimmer with Bcl-2. It antagonizes the action of Bcl-2 in the presence of various cell death signals and induces apoptosis (Wu et al, 1998). The expression of Bax protein was immuno-histochemically localized in 68% (27/40) cases of our study group; the positivity was detected in 50% of grade I; 70% of grade II; and in 75% of grade III. Bax protein expression was significantly different with lymph duct invasion (P<0.03) and ploidy pattern (P<0.002). Also, the incidence of Bax gene expression was increased with increasing of apoptosis. These findings suggest that Bax gene expression delays progression of the disease by promoting apoptosis (Charalambous et al, 2000). However, Bax promotes cell death through more than one mechanism, directly by Bax/Bax homodimerzation and indirectly by binding to and preventing Bcl-2 from fulfilling its function as a death repressor. Bcl-2 must be able to form heterodimers with Bax in order to function as a suppressor of cell death (Reed, 1997).

Bcl-2 and Bax are in an inverse relation in various organs, and cell death is controlled by their balance (Krajewski et al, 1994). Korsmeyer et al, (1993) proposed the hypothesis that the Bcl-2/Bax ratio determines the sensitivity of tissue to apoptosis inducing stimuli. Koshida et al, (1997) reported that the two proteins were distributed in a reverse pattern in gastric cancer tissue. This kind of imbalance between Bcl-2 and Bax occurring in the process of carcinogenesis may be responsible for the marked morphological differences in gastric cancers (Masutani et al, 2001). In the present study, Bcl-2(-) /Bax(+) patients showed apoptotic index (AI) higher than that in Bcl-2(-)/ Bax(-) patients. One potential interpretation of these results is that the lose of both Bcl-2 and Bax expression is indicative of a highly deregulated state of apoptosis control and thus reflects more aggressive tumor behavior (Charalambous et al, 2000). These results indicate that apoptosis seems to be more dependent on Bax as reported in Masutani et al, (2001). Proposed mechanisms include the formation of heterodimers between Bcl-2/Bcl-xL and Bax, which would interfere with the availability and translation of the Bax protein from the cytoplasm to the mitochondria. Alternatively, counteracting ion channels at the level of the mitochondria may help to explain the antagonistic nature of these proteins (Lan and Lu, 2004).

Ikeda et al, (1998) observed that the progression of gastric cancer is defined by a gradual increase of proliferation activity and constant occurrence of apoptosis. Furthermore, the naturally occurring apoptosis is induced predominantly via p53 gene independent pathway. The half life of the mutant protein p53 is prolonged when p53 gene is mutated and then it is easily can be detected by immunohistochemical methods. In the present study, p53 protein was detected in 63% (25/40) of patients with gastric cancer; the positivity was correlated with malignancy grades where 25% (2/8) of grade I; 65% (13/ 20) of grade II; and 83% (10/12) of grade III (P<0.009). These data may indicate that the mutation of p53 is an event in the late gastric cancer as reported by Xu et al,

(2001).

In the present study, cases positive for apoptosis showed increased expression of p53 and decreased expression of Bcl-2 when the activity of apoptosis was increased (at severe AI). These results may be explained on the basis that, the p53 gene promotes apoptosis in cells with genetic damage. While, Bcl-2 is anti-apoptotic gene, it is therefore possible that the balance between p53 and Bcl-2 may be significant (Kesari et al, 1997). In conclusion, these data suggests that Bcl-2 family members may play a modulating role in mammalian cell death machinery. Therefore, analysis of apoptosis and its related gene products, Bcl-2, Bax and p53 may help in studing the tumor aggressiveness and to predict their behavior.

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