

Correlation between Serum Anti-Apoptotic Bcl-2 Level and its Immunohistochemical Expression in Relation to Apoptosis in Gastric Cancer

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Abstract

Introduction: Gastric cancer (GC) remains a major public health problem worldwide being the third commonest cause of cancer death worldwide. Many recent studies focus on the immunohistochemical evaluation of Bcl-2 expression and its serum expression and its prognostic significance in gastric cancer each one separately. We conducted our study to determine the correlation between serum Bcl-2 antigen and gastric carcinoma, to investigate whether serum Bcl-2 concentrations can be used as marker for immunohistochemical determination of Bcl-2 alterations in gastric cancer patients and examine the association between its expression and other clinicopathological parameters.

Methods: Our study conducted in Forty-five consecutive patients with gastric cancer underwent gastrectomy in Mansoura Gastroenterology Center. ELISA was used for the estimation of serum Bcl-2 levels in patients with different stages of gastric cancer. Immunohistochemical localization of Bcl-2 antigen was performed on formalin-fixed, paraffin-embedded tissue block; Bcl-2 expression was detected from mild to moderate apoptotic index (AI).

Results: Positive serum Bcl-2 expression was found in 13/45 patients (28.9%). Bcl-2 protein was immunohistochemically localized in the cytoplasm of 45% (18/40) of gastric cancer patients. Total apoptosis positivity in Bcl2 detected immunohistochemically was detected in 45% (18/ 40) of cases; while total apoptosis positivity in serum expression of Bcl-2 was detected in 28.9% (13/ 45).

Conclusion: our current study demonstrated that the formation and growth of cancer is a complex process that requires further research in correlation with the results assessed between serum and immunohistochemical expression of Bcl-2 and with its role in the process of apoptosis.

Keywords: Bcl-2; Apoptosis; Gastric cancer, Serum Bcl-2; Immunohistochemistry

Introduction

Gastric cancer (GC) remains a major public health problem worldwide being the third commonest cause of cancer death worldwide, with almost three quarters of a million deaths annually [1]. Several different types of cancer can arise in the stomach, including adenocarcinoma, lymphoma and leiomyosarcoma, but adenocarcinoma is by far the most common. Two types of gastric adenocarcinoma [intestinal-type and diffuse-type] can be differentiated histologically [2]. The proliferative activity and the apoptosis of premalignant and neoplastic tissue have been studied extensively.

Apoptotic disorders underlie carcinogenesis. The antiapoptotic Bcl-2 has a major role in gastric cancer [3,4]. The assessment of its expression in gastric cancer cells in relation to morphological and histological factors help elucidate the formation and growth of various forms of gastric cancer. Apoptosis is a physiological programmed cell death that plays a major role in the process of carcinogenesis. It is known as one of the most important systems which control the number of cells in tissues [2]. Apoptosis is regulated by a variety of genes including p53 and Bcl-2 which may play an important role to keep the homeostasis of tissue dynamics [5-9].

Bcl-2 is defined as a new class proto-oncogene and can block cell death without affecting proliferation [10]. The anti-apoptotic Bcl-2 Family plays a pivotal role in the protection against DNA damage-induced apoptosis [11]. The expression of Bcl-2 has been associated with many human cancers;

including gastric cancer. The presence of Bcl-2 in cancer patients may be associated with poor prognosis and metastasis [12,13].

Bcl-2 expression in cancer tissue is mainly assessed indirectly by immunohistochemistry. Immunohistochemical determination of Bcl-2 has been found to correlate with apoptotic activity in cancer tissue [2,3], however its detection necessitates the availability of cancer tissue from a surgical specimen or biopsy, is subject to the methodology used for the preparation of specimens and interpretation of findings [4,9], and cannot be easily performed serially as the disease progresses with formation of metastases. However, despite its limitations, Bcl-2 immunohistochemistry represents a useful prognostic factor [5,6], in selected categories of patients with colorectal cancer (CRC) [7,14].

Bcl-2 can be measured by enzyme linked immunosorbent assay in serum samples from patients with gastric carcinoma and significantly raised compared with negative controls. Although tissue Bcl-2

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estimation is a well-established procedure and its association with the invasiveness of the tumor and prognosis has been widely studied, serum Bcl-2 estimation has not been studied adequately.

We conducted this study to determine the correlation between serum Bcl-2 antigen and gastric carcinoma, and to investigate whether serum Bcl-2 protein concentrations can be used as marker for immunohistochemical determination of Bcl-2 alterations.

Material and Methods

Tumor samples

Forty-five consecutive patients with gastric cancer (29 males and 16 females) underwent gastrectomy in the Gastroenterology Center, Mansoura University, Mansoura, Egypt. The study was conducted in collaboration with the faculty of Medicine, Umm Al Qura University, Mekah city. The youngest patient was 20 yr old, the oldest 76 yr, with median age of the series was 49.9 ± 12.96 years. A written consent was obtained from each patient included in the study to use the samples and clinical data for research purposes after being informed about the nature of the study. The study protocol conforms to the most recent ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by local ethical committee. It was carried out during surgical resections for patients with gastric carcinoma either epithelial, stromal or even NHL underwent a potentially curative total or partial gastrectomy. All the information was reviewed such as age, sex, tumor location, tumor size, histological proliferation, clinical stage, depth of invasion, and metastasis. The current study was pursued on those patients prior to treatment including neo-adjuvant, radiotherapy and/or chemotherapy. Negative controls were obtained from fifteen of normal serum and gastric mucosa samples. Tissue specimens were stored at -70°C until used histologically diagnosed by the microscopic examination of hematoxylin and eosin stained sections.

Measurement of serum Bcl-2 antigen in serum gastric cancer patients using ELISA

Serum concentrations of the Bcl-2 protein were analyzed by Bcl-2 ELISA assay kit provided by oncogene research products (Oncogene Science, Cambridge, UK). The Bcl-2 ELISA is a "sandwich" enzyme immunoassay employing mouse monoclonal antibodies. Briefly, microtiter wells were precoated with mouse monoclonal antibodies specific for most native mammalian mutant Bcl-2 protein. Aliquots (50 μL) of serum sample were added to each well, and were incubated at room temperature for 2 hours. After washing the wells, 100 μL FITC conjugated detector monoclonal antibody were pipetted into each well, covered with a plate sealer and were incubated at room temperature for 30 minutes. After washing the wells, 100 μL horseradish peroxidase-conjugated anti-FITC antibody was added to each well, and was incubated in the dark at room temperature for 1 hour. After washing again, the wells were incubated with 100 μL chromogenic tetramethylbenzidine (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 was quantified by examining its absorbance at 450 nm using a spectrophotometer.

The concentration of mutant Bcl-2 protein in the serum was then determined by comparison against a standard curve generated from a known concentration of mutant Bcl-2 protein (0 U/ml, 5.12 U/ml, 12.8 U/ml, 32 U/ml, 80 U/ml and 200 U/ml). By this approach, this assay can be seen to have a limit of detection of 5.12 U/ml and generate a linear standard curve up to 200 U/ml, with cross reactivity with normal Bcl-2 according to the manufacturer (Oncogenic Science, Cambridge, UK).

Immunohistochemistry

Immunohistochemical localization of Bcl-2 antigen was performed on formalin- fixed, paraffin-embedded tissue blocks that were cut on neoprene-coated slides. The immunostaining was performed using the avidin-biotin complex (ABC) method and an automatic autostainer (CODE-ON Immuno/DNA slide stainer; Biotek solution, Santa Barbara, CA). Slides were deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 min. Antigen retrieval was performed for 15 min using Biogenex Antigen Retrieval Citra solution in 90°C water bath for 30 min. The slides were allowed to cool for 20 min before continuing. Slides were then blocked by normal goat serum for 5 min at 37°C . The monoclonal antibody was applied overnight in humid medium at room temperature followed by the biotinylated secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min at room temperature as chromogen, slides were counterstained with Mayer's hematoxylin, dehydrated, and covered by cover slips. In negative control slides, the same system was applied with replacement of the monoclonal antibody by diluted normal bovine serum. All slides were evaluated blindly without knowledge of the clinical outcome. Sections were considered positive if more than 5% of tumor cells were stained. Cytoplasmic immunostaining was scored for Bcl-2 expression regarded as negative (score=0) and positive (score > 0) [5] that describes the diffuse and focal patterns of Bcl-2 immunoreactivity, considering the diffuse pattern representing the mutated Bcl-2 protein and the focal one for the wild type Bcl-2 product.

Apoptosis detection

Apoptotic bodies were seen as single dark-stained roundish or pyknotic nuclear remnants, two or more nuclear fragments with uneven sizes and cytoplasmic eosinophilia located within or between adjacent tumor cells. Scores were given for each case based on the apoptotic bodies/HPF as follows: negative, 0; mild, 1–2; moderate, 3–5; and severe >5. Areas with much necrosis were excluded [6,7].

Statistical analysis

All statistical analyses were done using SPSS for Windows version 11.0 and statistical significance was indicated at a two-sided *p* value <0.05. Numerical data were expressed as mean standard deviation (SD). The levels of markers were analyzed by analysis of variance. Correlations between serum Bcl-2 protein marker and clinicopathological variables data were evaluated by Pearson's correlation coefficient. A *p* value <0.05 was considered significant.

Results

Serum Bcl-2 protein

The expression of Bcl-2 was frequent in gastric cancer patients (40.6 to 86.6 units/ml, with average 51.3 ± 9.2 units/ml) compared with those in healthy blood donors as a negative control, 44.9 ± 4.2 unit/ml ($p=0.004$). The cut off value for the serum Bcl-2 protein concentration in the stomach cancer cases was defined as 53.4 unit/ml. Therefore, serum Bcl-2 concentration above 53.4 unit/ml was defined as positive and those below as negative.

Positive serum Bcl-2 expression was found in 13/45 patients (28.9%). The positive Bcl-2 protein was more frequently expressed in cancer cells in patients with gastric cancer (61.6 ± 10.9 unit/ml) than in the negative group (47.1 ± 3.4 unit/ml), the difference being statistically significant ($p<0.0001$) (Figure 1).

Clinicopathological parameters were assessed and correlated with serum Bcl-2 expression, and it was not significantly different with tumor type, patients' gender, age, tumor size, depth of invasion, stromal reaction and tumor site ($p>0.5$) (Table 1). Regardless of tumor stages, No statistically significant differences were observed in the positive expressions of Bcl-2 and tumor stages ($p=0.78$), (Figure 2).

Bcl-2 Immunostaining

Bcl-2 expression was positive in 45% (18 of 40) of in gastric cancer tissues and the rest of tumors (22 cases) were negative for Bcl-2 immunostaining. Bcl-2 staining was observed in the cytoplasm and cytomembrane of carcinoma cells (Figure 3). 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 ($P<0.02$) (Table 2). Mean Bcl-2 concentrations were 54.13 ± 13.52 in

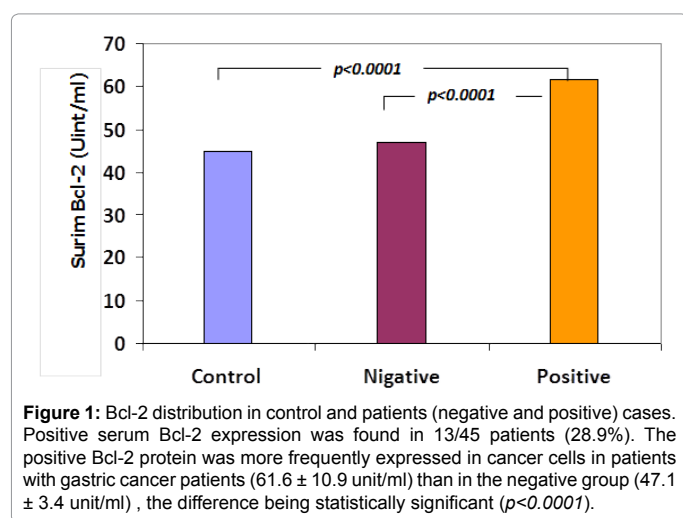


Figure 1: Bcl-2 distribution in control and patients (negative and positive) cases. Positive serum Bcl-2 expression was found in 13/45 patients (28.9%). The positive Bcl-2 protein was more frequently expressed in cancer cells in patients with gastric cancer patients (61.6 ± 10.9 unit/ml) than in the negative group (47.1 ± 3.4 unit/ml), the difference being statistically significant ($p<0.0001$).

	Serum bcl-2 antigen (ELISA)			P. value
	Total No. (%)	Positive Bcl-2 No. (%)	Negative Bcl-2 No. (%)	
Mean Age \pm SD	49.9 \pm 2.96	48.5 \pm 12.8	50.6 \pm 13.4	0.88
Sex				0.1
Male	29 (64.4%)	6 (20.7%)	23 (79.3%)	
Female	16 (35.6%)	7 (43.7%)	9 (56.3%)	
Size				0.19
<5	17 (37.8%)	3 (17.6%)	14 (82.4%)	
>5	28 (62.2%)	10 (35.7%)	18 (64.3%)	
Site				0.17
Antrum	25 (55.6%)	8 (32.0%)	17 (68.0%)	
Body	13 (28.9%)	4 (30.8%)	9 (69.2%)	
Cardia	7 (15.6%)	1 (14.3%)	6 (85.7%)	0.19
Gross (shape)				
Ulcerating	27 (60.0%)	8 (32.0%)	19 (70.4%)	
Infiltrating	9 (20.0%)	4 (30.8%)	8 (88.9%)	
Polypoi	9 (20.0%)	1 (14.3%)	5 (55.6%)	0.8
Type				
Epithelial tumor	37 (82.2%)	8 (29.6%)	27 (73.0%)	
Stromal tumor	3 (6.7%)	1 (11.1%)	1 (33.3%)	
NHL	5 (11.1%)	4 (44.4%)	4 (40.0%)	0.12
Grade				
Low	10 (22.2%)	3 (30.0%)	7 (70.0%)	
Intermediate	23 (51.1%)	4 (17.4%)	19 (82.6%)	
High	12 (26.7%)	6 (50.0%)	6 (50.0%)	
Total	45 (100%)	13 (28.9%)	32 (71.1%)	

Table 1: Clinicopathological parameters against serum Bcl-2 antigen by ELSIA.

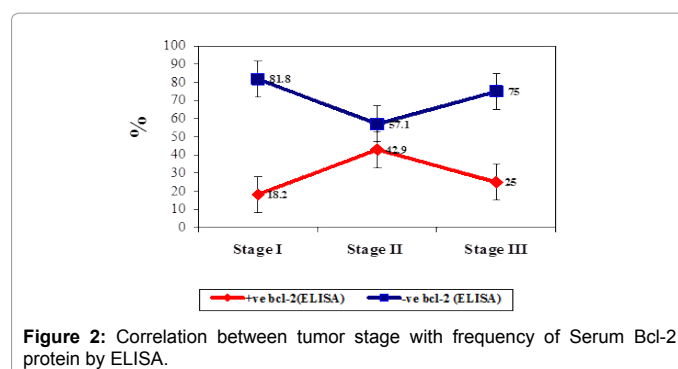


Figure 2: Correlation between tumor stage with frequency of Serum Bcl-2 protein by ELISA.

	bcl-2 expression (I.H)			P value
	Total No. (%)	Positive bcl-2 No. (%)	Negative bcl-2 No. (%)	
Mean Age \pm SD	50.7 \pm 13.2	47.4 \pm 12.7	53.2 \pm 13.2	0.47
Sex				0.6
Male	24 (60.0%)	10 (41.7%)	14 (58.3%)	
Female	16 (40.0%)	8 (50.0%)	8 (50.0%)	
Size				0.14
<5	13 (32.5%)	8 (61.5%)	5 (38.5%)	
>5	27 (67.5%)	10 (37.0%)	17 (63.0%)	
Site				0.31
Antrum	22 (55.0%)	12 (54.5%)	10 (45.5%)	
Body	13 (32.5%)	5 (38.8%)	8 (61.5%)	
Cardia	5 (12.5%)	1 (20.0%)	4 (80.0%)	0.5
Gross (shape)				
Ulcerating	22 (55.0%)	9 (37.5%)	15 (62.5%)	
Infiltrating	7 (17.5%)	4 (57.1%)	3 (42.5%)	
Polypoid	9 (22.5%)	5 (55.6%)	4 (44.4%)	0.15
Type				
Epithelial tumor	32 (80.0%)	12 (37.5%)	20 (62.5%)	
Stromal tumor	3 (7.5%)	2 (66.7%)	1 (33.3%)	0.026
NHL	5 (12.5%)	4 (80.0%)	1 (20.0%)	
Grade				
Low	8 (20.0%)	7 (87.5%)	1 (12.5%)	
Intermediate	20 (50.0%)	7 (35.0%)	13 (65.0%)	
High	12 (30.0%)	4 (33.3%)	8 (66.7%)	
Total	40 (100%)	18 (45.0%)	22 (55.0%)	

Table 2: Clinicopathological parameters against Bcl-2 expression detected by immunohistochemical (I.H)

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 ($P<0.03$). There was no correlation between Bcl-2 and other pathological parameters, such as age, gender, histological type, tumor location, or tumor size (Table 2). Also, there was no significant association between Bcl-2 expression and tumor stage ($p=0.9$) (Figure 4).

Correlation between serum Bcl-2 protein concentration and immunostaining

The correlation between serum Bcl-2 protein concentration and immunohistochemical staining for Bcl-2 protein was analyzed in 40 matched cases of gastric cancer (Table 3). Positive serum Bcl-2 concentrations were found in 10 (55.6%) of 18 positive immunostained cases, but in only three (13.6%) of 22 negative immunostained cases ($p<0.002$) (Figure 3). In addition, serum negative Bcl-2 was found in 19 (86.4%) of 22 cases negative for Bcl-2 protein by immunostaining (Table 3). Positive immunostaining for Bcl-2 protein was found in 10

(76.9%) of 13 cases positive for Bcl-2 protein in serum and in 8 (29.6%) of 27 cases negative for serum Bcl-2 antigen (Figure 4 and Table 3).

Apoptotic index

Expression of Bcl- 2 was increased stepwise from mild to moderate apoptotic index (AI) but negative expression was observed at severe AI. Total apoptosis positivity in Bcl-2 detected immunohistochemically was detected in 45% (18/40) of cases with gastric cancer; while total apoptosis positivity in serum expression of Bcl-2 was detected in 28.9% (13/45) of cases with gastric cancer (Table 4). However, there is no significance between AI with clinicopathological variables.

Discussion

In addition to known conventional prognostic factors such as the depth of tumor invasion, the number of involved lymph nodes and the involvement of resection margins, a plethora of markers have been suggested as possible prognostic factors in patients with gastric cancer, including the apoptosis inhibitor Bcl-2, tumor suppressor genes.

It is well established that apoptosis is deregulated during cancer development and progression. The anti-apoptosis Bcl-2 protein was over-expressed in various malignancies at mRNA and protein levels [15,16]. In GC, previous studies have shown that expression of Bcl-2 gene suppresses the cellular proliferative activity and is correlated with less aggressive biological behavior [17-19].

In this study, we investigated the serum level and immunohistochemical expression of Bcl-2 protein in 40 gastric

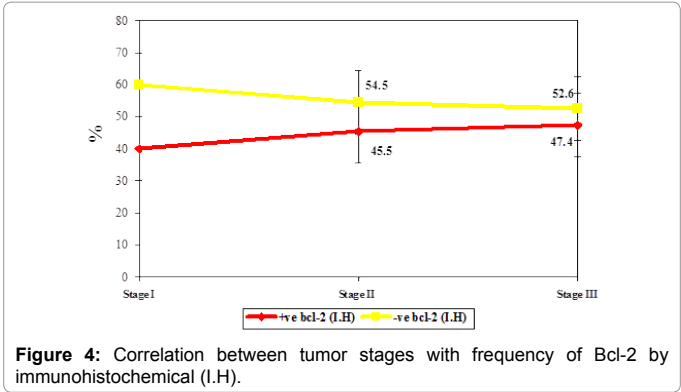


Figure 4: Correlation between tumor stages with frequency of Bcl-2 by immunohistochemical (I.H).

	Negative No.(%)	Mild No.(%)	Moderate No.(%)	Marked No.(%)	<i>P value</i>
Bcl-2 expression (I.H)					
Positive Bcl-2	1 (25.0%)	8 (47.1%)	9 (47.4%)	18/40 (45.0%)	0.69
Negative Bcl-2	3 (75.0%)	9 (75.0%)	10 (52.6%)	22/40 (55.0%)	
Serum bcl-2 antigen					
Positive Bcl-2	2 (28.6%)	4 (22.2%)	7 (35.0%)	13/45 (28.9%)	0.57
Negative Bcl-2	5 (71.4%)	14 (77.8%)	13 (65.0%)	32/45 (71.1%)	

Table 4: Correlation between Apoptotic index (AI) (negative, mild, moderate, marked) serum Bcl-2 protein expression in 45 patients and immunostaining in 40 patients with gastric cancer.

	Bcl-2 immunostaining		
	Negative Bcl-2 No.	Positive Bcl-2 No.	Total Bcl-2 No.
SerumBcl-2 protein			
Negative	19	8	27
Positive	3	10	13
Total	22	18	40

Note: Positive serum Bcl-2 concentration were found in 10 of 18 (55.6%) positive immunostaining cases, but in only 3 out of 22 (13.6%) negative immunostaining.

Table 3: Correlation between serum Bcl-2 protein concentration and immunostaining in 40 patients with gastric cancer

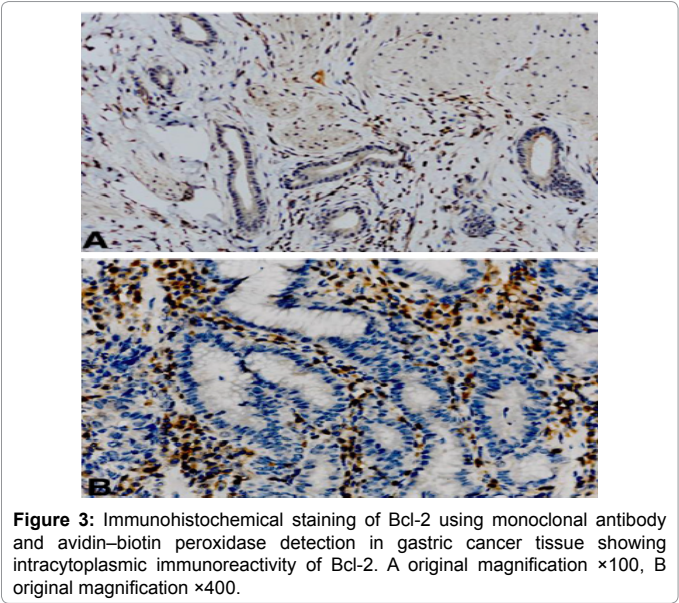


Figure 3: Immunohistochemical staining of Bcl-2 using monoclonal antibody and avidin-biotin peroxidase detection in gastric cancer tissue showing intracytoplasmic immunoreactivity of Bcl-2. A original magnification ×100, B original magnification ×400.

carcinomas and the results were compared with the clinicopathological data of these patients. Bcl-2 protein was detected by ELISA assay in the sera of 28.9% (13/45) 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 [20]. The expression of Bcl-2 is a phenomenon that occurs in the early period in the development of gastric cancer. These results are in agreement with Nasif et al., who reported that Bcl-2 protein was detected by ELISA assay in the sera of 32.5% (13/40) [21]. Mean serum concentration of Bcl-2 positive was elevated significantly ($P<0.0001$) in positive cases compared to negative ones. Also these results are coincident with those reported by Mohamed El-Shahat et al. who's stated that Bcl-2 expression is elevated in liver of cirrhotic patients and this increase may correlate with the development of HCC [22]. Also our results were agreed with other previous results of Athanassios et al. who reported that Bcl-2 protein was expressed in 67% of gastric adenocarcinomas examined [23]. 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 [24].

According to tumor size; there was a significant negative correlation with the tumour size. These results are coincident with the results reported by LIU Hai-Feng, et al. and Jianghong Wu et al. reported that there was no significant relationship between Bcl-2 protein expression and tumor size respectively [25,26]. But Silviastri et al. found that Bcl-2 protein expression was related to the tumor size [27]. A significantly higher fraction of Bcl-2 positive cells was observed in small tumors than in large tumors. But these results was not confident with that reported by Jianghong Wu et al. who reported that There was no correlation between Bcl-2 and other clinicopathological parameters, such as age, gender, histological type, tumor location, or tumor size [26].

Positive staining for Bcl-2 expression was detected in 45% (18 of 40) of gastric cancer tissue. This proportion is similar to that in other studies as that reported in previous studies by Saegusa et al. and Muller et al. was found in 12.6% (13/103) and 11.4% (47/413) of cases, respectively [24,28]. These results was also comes coincident with that reported by Jianghong Wu et al. who found that Bcl-2 expression was positive in 21.2% of all gastric cancer tissues [26]. Bcl-2 staining was observed in the cytoplasm and cytomembrane of carcinoma cells.

A Significant correlation was detected between positive Bcl-2 and tumor grade ($p < 0.026$). This is coordinated with the results of Dorra Ben Ayed et al. and Ben Ayed-Guerfali et al. who reported that there was a significant association between Bcl-2 expression and grade of tumor [29,30]. Also this result is agreed with Lukyanova et al. who reported that Bcl-2 is differently expressed in epithelial ovarian carcinoma varying by histological grade as well as biological features which could be useful for both prognosis and therapy correction [31]. But there was no significant association between Bcl-2 expression and tumor stage ($p = 0.9$).

One characteristic feature of cancer is continuous growth. An important feature to assess the clinical behavior of tumors is to assess the balance between the proliferative activities and the apoptosis processes [29]. The rates of cell proliferation and cell death may determine the speed of this growth [32]. The inability of cells to undergo apoptosis may advance cancer development, both by allowing dividing cells to accumulate and by not eliminating genetic mutants that may harbor enhanced malignant potential. Total apoptosis positivity in Bcl-2 detected immunohistochemically was detected in 45% (18/ 40) of cases with gastric cancer, while total apoptosis positivity in serum expression of Bcl-2 was detected in 28.9% (13/ 45) of cases with gastric cancer; the total negativity was recorded in 71.1% (32/ 45). Moderate to marked AI was detected in 9 (47.4%). While moderate to marked expression was detected in 7 (35%) of positive serum Bcl-2 expression. These results come coincident with the results previously reported by Athanassios et al. which revealed that none statistically significant correlation was found between Bcl-2 immunoreactivity and apoptotic body index and survival [23]. This indicates that the failure of the apoptotic process leads to an increase survival of cells with DNA damage. The mechanism may be involved in GC.

Conclusion

The positivity of Bcl-2 expression correlated with different groups of gastric cancer was studied in the current study. Immunohistochemical Bcl-2 expression is suggested to have a possible prognostic value with serum Bcl-2 expression on gastric cancer. In advanced-stage and high-grade gastric carcinomas indicates that Bcl-2 is involved in early stage of tumor development and constitutes an independent prognostic factor regarding the outcome of patients with gastric cancer. The presented results demonstrated that the formation and growth of cancer is a complex process that requires further research in correlation with the results assessed between serum and immunohistochemical expression of Bcl-2 and with its role in the process of apoptosis.

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