The Role of Helicobacter pylori CagA infection on the presence of Bcl-2 marker in gastric carcinoma by immune-histochemical analysis and *in situ* hybridization technique

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Abstract:

Background: Bcl-2 plays a major role in the process of apoptosis and their dysfunction underlies carcinogenesis.

Objectives: The study objective was to assess the expression of Bcl-2 in gastric cancer cells in correlation with clinic-pathological parameters and to determine the effect of *H. Pylori* infection on the expressions of Bcl2 in gastric adenocarcinoma.

Methods: Using immune-histochemical analysis and *in situ* hybridization technique were used to measure Bcl-2 protein and CagA *H. pylori* gene expressions respectively and its relation to clinic-pathological parameters were observed.

Results: The results revealed that a significant differentiation between Bcl-2 expression on the patients with gastric cancer and control group. Also we found thatBcl-2 expression significantly higher in intestinal type in comparison to that diffuse type (p<0.001). But no significant association between positive Bcl-2 expression and the presence of CagA-*H. pylori* in patients with gastric carcinoma (p>0.05).

Conclusion: Bcl-2 expression in gastric cancer patients was suggested of its association with gastric carcinoma especially intestinal type. Thus, detection of Bcl-2 markers might be a useful early diagnostic in gastric cancer. *H pylori* enhance Bcl-2 protein levels which cause deregulation of these apoptosis-associated genes that may play a role in the development of gastric adenocarcinoma.

Keywords: Bcl-2, *H. pylori* CagA, gastric carcinoma

Introduction:

Gastric cancer is a multifactorial disease[1]. *Helicobacter pylori* (*H. pylori*) infection and the associated protein dysfunctions, including anti-apoptotic Bcl-2 and several other pro-apoptotic proteins are essential risk factors in 65–80% of gastric cancers, this occur in only 2% of such infections[2,3]. *H. pylori* can cause chronic inflammation or the action of its virulence factors such as CagA (cytotoxin-associated gene A) [4], which potentially induce stomach cancer[5].

B-cell lymphoma 2 (Bcl-2) encoded in humans by the Bcl-2family regulator proteins that regulate cell death (proapoptotic) it or inhibiting it (antiapoptotic) Bcl-2 is specifically considered as an important antiapoptotic protein and is thus classified as an oncogene[6].

The Bcl-2 proto-oncogene; initially discovered at the chromosomal translocation t(14;18)in human follicular lymphomas and b-cell lymphomas, is a known inhibitor of apoptosis alterations[7]. The Bcl-2 gene, located on chromosome 18, encodes a 25kd protein that localizes to mitochondrial membrane, nuclear envelope and endoplasmic reticulum although alterations of Bcl-2 expression have been most extensively studied in hematopoietic tissue[3]. IncreasedBcl-2 expression has been described in several epithelial tumors including carcinomas of the breast; lung and colon controversy still exists with regard to the prognostic value of Bcl- 2 expression in gastric cancer[8].

The pathogenicity of *H. pylori* may be increased by genes of the cag pathogenicity island. About 50-70; of *H. pylori* strains in western countries carry the Cag pathogenicity island (CagA)[9]. Patients infected with strains caring the Cag A have a stronger inflammatory response in the stomach and are at a greater risk of developing peptic ulcers of stomach cancer than those infected with strains leaking the island[10].

Materials and Methods:

The studied 56 case of patients with gastric cancer (GC) including, 31 men and 25 women ranged between 46 and 79 years (mean 66.3 years), who attended to Baghdad Teaching Hospital, AL-Yarmouk Teaching Hospital and Gastroenterology and Hepatology Teaching Hospital. 34 cases out of, 56 cases from GC have *H. pylori*. The control group included 34 cases with normal mucosa (N) and non-infected with *H. pylori*. All cases of chronic gastritis were endoscopically resected biopsies while all the studied gastric cancer cases were surgically resected gastrectomy specimens or endoscopically resected biopsy. Patients were diagnosed over the period of study from January 2014 to December 2014, was eligible for this study.

Classification, grading and both pathological staging and stage grouping of GC cases were performed according to WHO. Patients were divided into two groups according to the age, those ≥60 years and others <60. *H. pylori* were confirmed by the rapid urease test and histology. None of these patients was taking aspirin or NSAID. Scoring was done by two independent investigators and the mean score was taken in each case. Antral biopsies were taken from the greater and lesser curvature within 2 to 3cm from the pylorus for assessment of Bcl-2 staining and *H. pylori* CagA gene.

Each specimen was fixed in 10%phosphate-buffered formalin immediately after resection, embedded in paraffin and cut into 4μm-thick

sections for immune-histochemical study and routine histological examination.

In situ hybridization

The biopsy specimens were embedded in paraffin and stained with haematoxylin-eosin (H&E) and Giemsa stained for *H. pylori* determination and diagnostic as chronic gastritis. In situ hybridization (ISH) for detection of *H. pylori* / CagA gene, which is a technique[11], makes use of the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell. For detection of this markers, the biotinylated DNA probe hybridize to the target sequence (*H. pylori* / CagA mRNA sequence) then a streptavidin-AP (streptavidin-alkaline phosphatase) Conjugate is applied followed by addition of the substrate promo-chloro-indolyl-phosphatel/nitro-blue tetrazolium (BCIP/NBT) which yield an intense blue-black signal appears at the directly specific site of the hybridized probe. This strepteividin-Ap conjugate like the biotinylated probe and highly sensitive detection method. We used Biotin-Labeled DNA probe for *H. pylori*/ CagA (8 µg/10015 ML) litter dd H_2O. Probe size: 349 bp (Maxim Biotech, Inc., USA).

Scoring:

Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in both positive test tissues. A scoring system that includes evaluation of the staining percentage of stained gastric cells was employed for the expression of CagA gene of *H. pylori*. Tissues were regarded as *H. pylori* CagA positive when their ISH signaling scores were < 10[11].

Immunohistochemistry

Mucosal biopsies were immunostaining with the use of universal Dako Cytomation streptavidin-biotin system purchased from Dako Cytomation (USA) Immunohistochemistry detection kit. The mouse anti-human monoclonal antibodies Bcl-2 protein (code No. / c-2: sc-7382) (santacruz, cut USA). The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary antibody will form a complex with the peroxidase-conjugated streptavidin and by adding the substrate, which contains 3, 3-diaminobenzidine (DAB) in a chromogen solution, counterstained with Mayer hematoxylin, and mounted.

Evaluation of immunostaining

Counting the number of positive cells which gave brown cytoplasmic staining system under light microscope, the extent of the IHC signal was determined in 10 fields (X100 magnification).

To evaluate Bcl-2 expression, a score was established. Percentage of positive cells (0 = 0% immunopositive cells, 1 = <5% of cells stained, 2 = 20% of cells stained, and 3 = 20 -50% of cells stained and 4= >50% of cells stained[12].

Statistical analysis; Data were analyzed by SPSS. The associations between expressions of Bcl-2 and *H. pylori* CagA gene, clinic-pathological parameters were assessed by the Chi-square test. The Bcl-2 proportional hazard model was used for multivariate analysis of prognostic factors. $P<0.05$ was considered statistically significant.

Result:

Clinical and pathological data for the studied gastric cancer patients are represented in (Table-1). The results do not show a significant difference between Bcl-2 expression and the sex and age of the patients with gastric cancer ($p>0.05$).

The Bcl-2 immunoreactions have become significantly positive more frequently in gastric carcinomas of intestinal type (76.92%), in comparison to the carcinomas of diffuse type (23.07%). The expression of Bcl-2 protein was heterogeneous dark brown staining in the tissue shown in figure 1.

Expression of Bcl-2 in *H. pylori* positive group (71.79%) was significantly higher than that *H. pylori* negative group (28.20%) ($P<0.01$). Levels of Bcl-2 protein between $CagA^{+}$*H. pylori* infection group (53.84%) and $CagA^{-}$*H. pylori* infection group (46.15%) did not show any significant difference (Table 2, Figure 2).

In Table 3, Chi-square test of significant was conducted to examine the association between Bcl-2 expression in normal mucosa non-infected patients (control) and gastric cancer with *H. pylori*-infected patients. It was found that highly significant association ($P<0.001$) between them in the four scoring levels, the results showed that percentage of Bcl-2 were elevated in the gastric cancer with *H. pylori*-infected group than that in control group.

Table 1: The Bcl-2 expression in gastric cancer cells depending on clinic-pathological factors.

Variables	Bcl-2 expression		P value
	+ve No(%)	-ve No(%)	
Protein expression	39 (69.64%)	17 (30.35%)	<0.01
Age ≥60	16 (41.02%)	9 (52.94%)	>0.05 (NS)
<60	23 (58.97%)	8 (47.05%)	
Sex Male	19 (48.71%)	10 (58.82%)	>0.05 (NS)
Female	20 (51.28%)	7 (41.17%)	
Leurens classification Intestinal type	30 (76.92%)	5 (29.41%)	<0.001
Diffuse type	9 (23.07%)	12 (70.58%)	

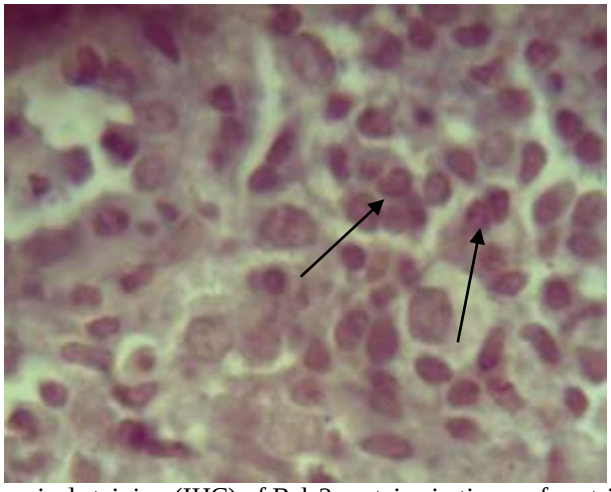

Figure 1: The Immuno-histochemical staining (IHC) of Bcl-2 proteins in tissue of gastric carcinoma. Staining by DAB chromogen (dark brown) counterstained with Haematoxylin. Positive Bcl-2 immunostaining (X400).

Table 2: The effect of *H. pylori* infection and Cag A gene expression on Bcl-2

Variables	**Bcl-2 expression**		**P value**
	+ve No(%)	**-ve No(%)**	
Protein expression	39 (69.64%)	17 (30.35%)	
***H. pylori* infection Present**	28 (71.79%)	6 (35.29%)	**<0.01***
Absent	11 (28.20%)	11 (64.70%)	
***H. pylori* CagA CagA+ve**	21 (53.84%)	7 (41.17%)	**>0.05 (NS)**
CagA-ve	18 (46.15%)	10 (58.82%)	

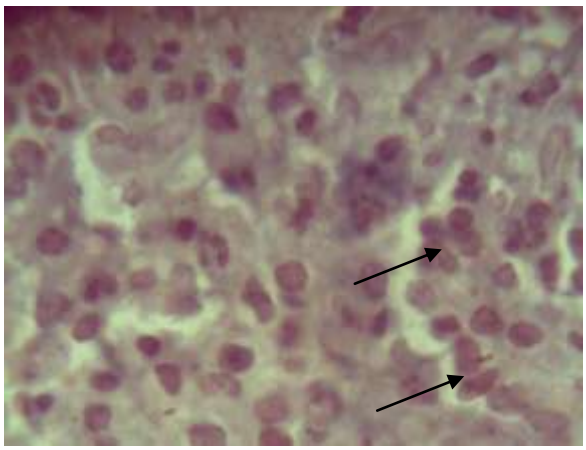

Figure 2: The Detection of CagA - *H. pylori* mRNA, in patients with gastric carcinoma by in situ hybridization technique. Tissue from patients withgastric carcinoma shows positive CagA - *H. pylori* by hybridization signals.

Table 3: The scoring of bcl-2 expression in non-infected patients (control) and *H. pylori*-infected patients with gastric cancer (GC).

Variable	**Score[a]**	**Groups**		**Total n=68**	***P* value**
		N(n=34) No. (%)	**GC(n=34) No. (%)**		
Bcl-2	**0**	22(64.70%)	0(0)	22	<0.001
	1	6(17.64%)	2(5.88%)	8	
	2	2(5.88%)	3(8.82%)	5	
	3	0(0)	11(32.35%)	11	
	4	0(0)	18(52.94%)	18	

Score[a]:0 = 0% immunopositive cells, 1 = <5%positive cells, 2 = <20% positive cells, and 3 = 20 - 50% positive cells and 4 > 50%positive cells.

Discussion:

In the present study, positive Bcl2 expression was found in more than two thirds of the study sample (69.64%) which approximately similar to the finding of a previous study which was conducted by Tsamandas *et al*, among 110 patients with gastric cancer in Greece where (67%) of the patients have positive expression of Bcl2 proteins[13], in turn Xinhao *et al*, reported that it was positively expressed in (65.4%) of patients with gastric cancer in their study from China[14].

While authors in other studies found extremely less positive Bcl2 expression as it was reported in (23.8%) in a study conducted by Yildirim *et al* in Turkey[15], and in (23%) by Smith et al in their study from the United Kingdom[16], Wu *et al* have reported that it was positive in only (21.1%) in their study from China[17], meanwhile it was not expressed in any of the patients studied by Van Der Woude *et al* from Netherlands[18].

This difference in the results might be due to using different methods to assess the expression of Bcl-2. Some studies assessed the Bcl-2 by PCR, so, one possible problem is the reliability of the Bcl-2 PCR assay because a correct design of primers is very important because of strain genomic diversity [8, 14]. Therefore, the different sets of Bcl-2 primers give different results, and this will be attributed to divergence in the primer target sequences[9, 14].

In the recent study neither age nor the gender of the participants shown to have significant associations with Bcl2 expression which was in concordance with previous study[13].

Regarding Lauren's classification, the current study found that; intestinal type was significantly higher among Bcl2 positive expression in comparison to the diffuse type; this was in the same line with what was reported by Gryko *et al*[18]. While it was in disagreement with previous study carried out United Kingdom by Triantafyllou *et al*[20] where they did not found similar association.

The resent study also found that *H. pylori* infection was presented in (71.79% and 35.3%) of those with positive and negative Bcl2 expression respectively with significant association between the infection and the expression; this was in agreement with two studies carried out in China by Zhang *et al* 2007[21,22] while the other study failed to find such association [19].

Zhang et al found a significant positive association between positive Bcl2 expression and the presence of CagA in patients with gastric carcinoma [20], this association was not found by our study neither in the study done in Poland *et al* [19], Lima *et al* also agreed with our results as they did not found such association [22]. 6A significant association of positive Bcl2 expression on the cells of the gastric mucosa in patients with gastric carcinoma, this was in agreement with the study done in Poland *et al*[19].

All above differences between the present study and others might be judged by the diversity in ethnicity, type of food, settings, sample size as well as the genetics of the studied samples.

Bcl-2 protein levels in *H. pylori* negative group in gastric adenocarcinoma tissues were significantly lower than in *H. pylori* positive group. Bcl-2 protein levels in *H pylori* positive group, CagA- and CagA+ infection group respectively increased but statistically not significant, suggesting that *H pylori* might promote expression of Bcl-2 in gastric adenocarcinoma [20].

Thus, suggesting that *H. pylori* infection، which is known to increase apoptosis, increases the expression of Bcl-2, and anti-apoptotic gene, giving chances for gastric carcinogenesis[22]. Also, explained by increased survival capacity acquired by cancer cells with high expression of antiapoptotic Bcl-2 or by possible changes in the protein profile in the course of cancer growth[17].

Conclusion:

In conclusion, this study showed that overexpression of Bcl-2 proteins is early events during gastric carcinogenesis. Thus, their immono-histochemical detection might be a useful indicator in gastric cancer.

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