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Evaluation of apoptosis along with BCL-2 and Ki-67 expression in patients with intestinal metaplasia

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ABSTRACT

The primary aim is to compare individuals with intestinal metaplasia (IM), chronic active gastritis (CAG), and normal gastric mucosa (NGM) in terms of apoptosis, proliferation, and Bcl-2 expression. The secondary aim is to determine whether these parameters are different between patients with and without gastric cancer in first-degree relatives. We enrolled 106 patients whose histopathological results were consistent with IM (*n*: 42), CAG (*n*: 51), or NGM (*n*: 13). Antral biopsies were immunohistochemically stained for Bcl-2 and Ki-67 expression. Apoptosis was detected using TUNEL assay. While no significant difference was determined between three groups with regard to apoptosis and Bcl-2 expression ($p > 0.05$), Ki-67 expression was significantly higher in the IM group when compared with the CAG and NGM groups (29.90 ± 22.87 *vs.* 18.18 ± 16.22 *vs.* 18.54 ± 20, respectively; $p = 0.012$). *Helicobacter pylori* was determined to increase apoptosis (49.3% *vs.* 25.7%, $p < 0.05$), nevertheless, it had no significant effect on proliferation and Bcl-2 expression. Bcl-2 and Ki-67 expression and apoptosis were not different among patients with and without a history of gastric cancer in first degree relatives. Although intestinal metaplasia cases demonstrate an increase in proliferation, no elevation is observed in apoptosis. This can be an important factor in the progression to gastric cancer.

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium that is helical in shape and is found in the stomach of more than half of the people worldwide. A causal relationship between *H. pylori* infection and gastric cancer has been consistently reported in many epidemiological and clinical studies [3,26,27].

Gastric cancer is generally assumed to develop in a stepwise fashion from chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia. The series of changes in gastric carcinogenesis, referred to as Correa's cascade [5], is often a consequence of *H. pylori* infection. In this context, atrophic gastritis and intestinal metaplasia are considered to be the precursors of gastric cancer, especially for the intestinal type of gastric cancer. However, all individuals with intestinal metaplasia do not progress to gastric cancer, and the factors influencing this progression need to be elucidated [16]. *H. pylori* infection is also causally related to the development of peptic ulcer. *H. pylori* infection results in two different clinical entities: duodenal ulcer or gastric ulcer/cancer. Duodenal ulcer patients usually display the antral predominant, non-atrophic type gastritis. In contrast, gastric cancer patients tend to develop multifocal or extensive atrophic gastritis of the corpus. Nevertheless, the mechanisms leading to these two different phenotypes are not fully understood [16].

Intestinal metaplasia (IM) is defined as the replacement of the gastric mucosa by an epithelium resembling the small bowel mucosa. Intestinal metaplasia arises from gastric stem cells transforming into small bowel epithelial cells such as absorptive cells, goblet cells, and Paneth cells, in place of proliferating into cells specific to the stomach [16].

Employing various histological and histochemical techniques, intestinal metaplasia can be classified into different subtypes. While several classification systems are used, the most widely accepted system has been proposed by Jass and Filipe [12]. In this classification, intestinal metaplasia is classified into complete and incomplete types.

The classification of intestinal metaplasia has prognostic significance. Complete or type I intestinal metaplasia carries a low risk for gastric cancer, on the other hand, type III (colonic) intestinal metaplasia has the strongest association with cancer [7,18].

Cell proliferation, which is essential for normal cell turnover, facilitates the action of carcinogens that target DNA when it is excessive. This may eventually result in carcinogenesis [21].

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Apoptosis, which is programmed cell death, plays a counter-regulatory role to cell proliferation in the maintenance of cell population. The alteration of the balance between apoptosis and cell proliferation is crucial in the development of gastric cancer [28].

Bcl-2 gene was initially identified due to its involvement in the chromosomal translocation t(14;18) observed in low grade B-cell lymphomas [2]. The excessive production of the protein prolongs cell life span despite apoptotic stimuli, and the protein is known to suppress apoptosis [1].

Ki-67 is an antigen associated with nuclear proliferation, and it is expressed during the growth and synthesis phases of the cell cycle, but not during the resting phase. This antigen provides information about the proportion of active cells in the cell cycle [8].

The aims of this study were:

(1) To compare individuals with intestinal metaplasia, chronic active gastritis, and normal gastric mucosa with regard to Bcl-2 expression, apoptosis, and proliferation.
(2) To determine whether or not Bcl-2 expression, apoptosis, and proliferation are different between the patients with and without history of gastric cancer in first-degree relatives.

Materials and methods

In this study, we enrolled 106 patients who had previously undergone upper gastrointestinal endoscopy due to dyspeptic symptoms and had been referred to our outpatient clinic of gastroenterology department with their biopsy results. Their histopathological results were consistent with intestinal metaplasia (IM), chronic active gastritis (CAG), or normal gastric mucosa (NGM). Informed consents were obtained from each patient, and the study was approved by the university ethical committee.

Fifty of the cases were male, 56 were female; age range was 18–81 years (mean age: 46.1 ± 14.5).

Patients with a history of *H. pylori* eradication treatment, gastric surgery, nonsteroid anti-inflammatory drug use, proton pump inhibitor or H2 receptor antagonist use, COX-2 inhibitor or acetylsalicylic acid use, antibiotic use during the previous month, exposure to corrosive substances, and those with malignancy and vasculitis were excluded from the study.

Family history of gastric cancer was questioned in all patients. The study population was divided into two groups: Patients with a history of gastric cancer in first-degree relatives and patients without a history of gastric cancer in first-degree relatives.

Histopathological analysis

Histopathological specimens of each patient were reassessed and categorized into intestinal metaplasia, chronic active gastritis, and normal gastric histopathology by a second pathologist who was blinded to the previous diagnoses. Histopathological sections of all the patients were evaluated according to the Updated Sydney System Score [6]. Moreover, immunohistochemical analysis was performed in order to determine Bcl-2, Ki-67, and apoptosis. *H. pylori* positivity was evaluated by Hematoxylin-Eosin. We performed subsequent extra thin-sections for the cases in which we did not detect *H. pylori* by Hematoxylin-Eosin. Thin sections were evaluated by Hematoxylin-Eosin with X100 objective.

Immunohistochemical staining was performed by an indirect immunoperoxidase method using streptavidin-biotin 3. The antibodies employed for Ki-67 and Bcl-2 were of IgG type and monoclonal character. For Ki-67 expression, RM-9106-R7 rabbit monoclonal antibody specific to the human Ki-67 nuclear antigen, which is expressed during the G1, S, M, and G2 phases of the cell cycle in all proliferating cells, was used (Lab Vision/NeoMarkers, Fremont, CA, USA). For Bcl-2 expression, we used MS-597-R7 specific to the human Bcl-2 oncoprotein (Diagnostic Biosystem, Pleasanton, CA, USA). The entire antibodies were in "ready-to-use" form. We used commercially available kits of biotinized binding (secondary) antibody, streptavidin-biotin complex, and AEC in ready-to-use form.

The sections with a thickness of four microns were deparaffinized by leaving them at 56 °C for 12 h in a sterilizer, before putting them in xylene for 30 min. They were hydrated in descending alcohol solutions (100%, 95%, and 90%). After washing them with tap water, they were placed in 3% hydrogen peroxide for 10 min in order to inhibit endogenous peroxidase. The sections were washed with PBS (phosphate buffered saline) solution (pH: 7.6) for 5 min twice and put in a microwave oven for 5 min in a 0.01 M sodium citrate buffer (pH: 6.0). They were washed with distilled water three times. Then the sections were placed in a non-immune serum for 20 min for protein block. Primary antibodies were applied so as to cover the sections, and they were kept at room temperature for 2 h. They were washed with PBS for 5 min twice and dried. Incubation with binding (secondary) antibody (Multi-species ultra streptavidin detection system-HRP, Signet, Massachusetts, USA) was performed at room temperature for 20 min. Sections were washed with PBS for 5 min twice.

Streptavidin-biotin complex was applied and allowed to set for 30 min. Then preparations were washed with PBS for 5 min twice. Incubation with DAB (diaminobenzidinetetrachloride, Novocastra, Newcastle-upon-Tyn, UK) was performed for 10 min, and the sections were again washed with distilled water for 5 min. Background staining was carried out with hematoxylin by rapid staining technique. The sections were dehydrated in ascending alcohol solutions by treating them 5 min in each (90%, 95%, and 100%). Finally, the sections were rendered more transparent by xylene and sealed with Entellan.

Tonsillar tissue was used as the positive tissue control for Ki-67 and Bcl-2. Nuclear staining was recognized as positive for Ki-67 and Bcl-2. Apart from the positive control, a negative control staining without any primary antibody was applied. Degree of staining was determined with a semiquantitative method.

Apoptosis associated with DNA fragmentation was detected by TUNEL assay. In this aim, ApopTag Plus peroxidase *in situ* apoptosis detection kit (Chemicon-S7101, Temecula, CA, USA) was used. The staining was carried out as per instructions for use. DAB was employed as the peroxidase substrate. Nuclear stainings with dark-brownish color were recognized as positive. Staining index was determined by counting nuclear staining in at least 1000 cells at randomly selected 10 microscopic fields under high magnification.

Statistical analysis

The data acquired in this study were analyzed by SPSS 12.0 package program. In normal distribution, comparison of two groups was performed by Student's *t*-test, whereas comparison of three groups was carried out by ANOVA. In cases where there was no normal distribution, comparison of two groups was performed by Mann–Whitney *U* test, whereas comparison of three groups was carried out by Kruskal–Wallis test. In categorical values, Pearson chi-square and Fisher's exact chi-square tests were used for dependency. $p < 0.05$ was recognized as presence of statistical significance and dependence, whereas $p > 0.05$ was recognized as absence of statistical significance and dependence.

Table 1
Demographic data of the study group.

	IM	CAG	NGM	
Number of the patients (*n*: 106)	42 (39.6%)	51 (48.1%)	13 (12.3%)	
Age (years)	52.02 ± 13.74	43.04 ± 14.21	39.23 ± 11.94	$p = 0.002$
Gender (male/female)	22/20	22/29	6/7	$p > 0.05$

IM, intestinal metaplasia; CAG, chronic active gastritis; NGM, normal gastric mucosa.

Table 2
Bcl-2, Ki-67 expression and apoptotic cell ratio based on histopathology results.

	IM (*n*: 42)	CAG (*n*: 51)	NGM (*n*: 13)	*p*
Bcl-2 expression rate (%)	45.2% (*n*: 19)	43.1% (*n*: 22)	15.4% (*n*: 2)	>0.005
Positive apoptotic cell ratio (%)	50% (*n*: 21)	37.2% (*n*: 19)	30.8% (*n*: 4)	>0.005
Ki-67 expression rate (%)	29.90 ± 22.87	18.18 ± 16.22	18.54 ± 20	0.03

IM, intestinal metaplasia; CAG, chronic active gastritis; NGM, normal gastric mucosa.

Results

In this study, 106 patients were included. They presented to our outpatient clinic because of dyspeptic complaints and were endoscopically diagnosed with intestinal metaplasia (*n*: 42), chronic active gastritis (*n*: 51), and normal gastric mucosa (*n*: 13) based on the biopsies acquired from the corpus and antrum. While 42 (39.6%) of 106 cases were intestinal metaplasia, 51 (48.1%) were chronic active gastritis, and 13 (12.3%) had normal gastric mucosa (Table 1).

There was no statistical difference between the three groups in terms of gender ($p > 0.05$). Mean age was statistically significantly higher in the IM group than in the CAG and NGM groups ($p < 0.05$) (Table 1). However, no statistically significant difference was found between the NGM and CAG groups with regard to age (39.23 ± 11.94 *vs.* 43.04 ± 14.21; $p > 0.05$). All the patients in the NGM group were *H. pylori*-negative. There was no statistically significant difference between the CAG and IM groups with regard to *H. pylori* infection (%76.5 *vs.* %76.2, respectively; $p > 0.05$).

Although Bcl-2 expression was higher in the IM and CAG groups than that in the NGM group, no statistically significant difference was observed between the three groups relative to Bcl-2 expression ($p > 0.05$) (Table 2). No statistically significant difference was determined between the *H. pylori* positive and negative groups in terms of Bcl-2 expression (%43.7 *vs.* %34.3, respectively; $p > 0.05$).

Although apoptosis tended to occur more frequently in the IM group than in the CAG and NGM groups, no statistically significant difference was observed between the three groups with regard to apoptosis detected by TUNEL assay ($p > 0.05$) (Table 2).

Apoptosis detected by TUNEL method was statistically significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients ($p = 0.02$) (Table 3 and Fig. 1).

There was a statistically significant difference between the IM, CAG, and NGM groups with regard to Ki-67 expression ($p < 0.05$). Ki-67 expression was statistically significantly higher in intestinal metaplasia patients (Table 1 and Fig. 2).

Paired comparison of the histopathological groups in terms of Ki-67 expression revealed no significant difference between the NGM and CAG groups ($p > 0.05$), but demonstrated a statistically significant difference between the CAG and IM groups in favor of the IM group (18.18 ± 16.22 *vs.* 29.90 ± 22.87, respectively; $p = 0.012$).

Table 3
Ratio of positive apoptotic cells relative to *H. pylori* presence.

	H. pylori positive (*n*: 71)	*H. pylori* negative (*n*: 35)	*p*
Positive apoptotic cells ratio (%)	49.3% (*n*: 35)	25.7% (*n*: 9)	<0.005

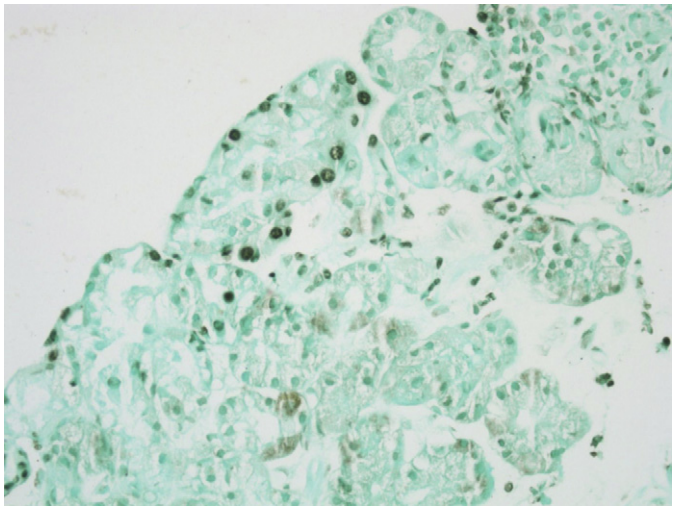

Fig. 1. Detection of apoptosis by TUNEL assay in a patient with *H. pylori* infection.

There was no significant difference between the *H. pylori*-positive and -negative patients in terms of Ki-67 expression (21 ± 17.02 *vs.* 23.79 ± 20.85, respectively; $p = 0.74$).

Comparison of the patients with and without history of gastric cancer in first-degree relatives showed no statistically significant difference with regard to presence of intestinal metaplasia (55.6% *vs.* 36.4%, respectively) ($p > 0.05$).

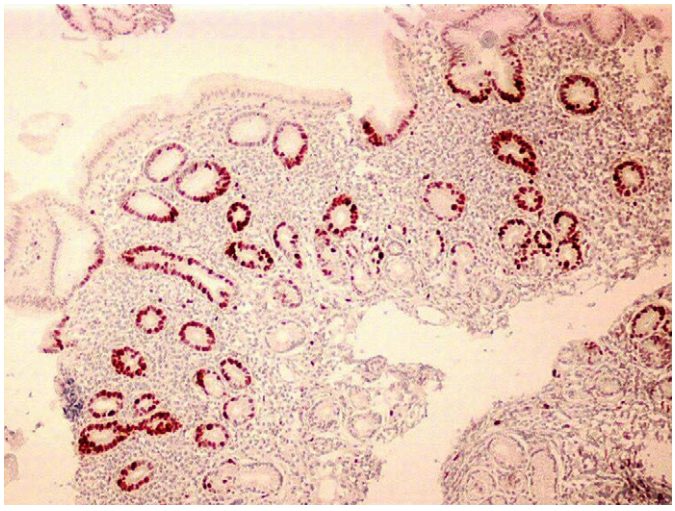

Fig. 2. Ki-67 expression in a patient with intestinal metaplasia.

As the study group was categorized into patients who have a first-degree relative with a history of gastric cancer (*n*: 18) and patients who do not have a first-degree relative with a history of gastric cancer (*n*: 88), no statistically significant difference was observed between these two groups with regard to apoptosis (%38.9 *vs.* %42, respectively), Bcl-2 (%55.6 *vs.* %37.5, respectively) and Ki-67 (%94.4 *vs.* %93.2, respectively) expression ($p > 0.05$).

Discussion

The development of gastric cancer, especially the intestinal type, is a stepwise process, beginning from chronic gastritis to atrophy, intestinal metaplasia, dyplasia, and eventually invasive cancer [5].

H. pylori, a common culprit causing chronic gastric disorders, has been classified as a class I gastric carcinogen [11].

Jass and Filipe proposed a classification for intestinal metaplasia [12], which has been widely accepted: complete (type I) and incomplete (types II and III).

It is well-known that the risk of gastric cancer is related to the type of intestinal metaplasia. In a cohort study from Slovenia with a follow-up period of 10 years, patients who had intestinal metaplasia had a 10 times higher risk of developing gastric cancer on average when compared to those who do not have intestinal metaplasia [7]. Patients with type III intestinal metaplasia had a 4-fold higher risk of gastric cancer when compared to those with type I [7].

While some studies [14,20] found a positive correlation between *H. pylori* infection and apoptosis and proliferation of the cells of normal gastric epithelium, Leung et al. [15] found that apoptotic index is significantly lowered in *H. pylori*-associated intestinal metaplasia. On the other hand, Hoshi et al. performed a study in which they evaluated apoptosis by TUNEL assay and proliferation by Ki-67 immunohistochemical staining in gastric mucosa specimens with and without *H. pylori* infection, and no statistically significant difference was found between the two groups in terms of apoptosis and proliferation [10].

Wambura et al. showed that before *H. pylori* eradication, there was a higher proliferation index in intestinal metaplasia, whereas apoptosis was significantly higher in non-intestinal metaplasia. This was associated with a significantly lower apoptosis/proliferation ratio in intestinal metaplasia than in non-intestinal metaplasia. After successful eradication, apoptosis and proliferation decreased in both intestinal metaplasia and non-intestinal metaplasia [25].

It is generally agreed upon that apoptosis has a relatively low profile despite an ongoing increase in proliferation in intestinal metaplasia. The studies showing an increase in proliferation parallel to decrease in apoptosis in gastric cancer tissues infected with *H. pylori* support this view [9,24].

In our study, we deployed TUNEL assay for detection of apoptosis developing with DNA fragmentation. In view of all three groups, although apoptosis was highest in the IM group, no statistically significant difference was found between them. However, apoptosis was observed to be statistically significantly higher in patients with *H. pylori* infection than in those with no *H. pylori* infection. This was a result consistent with the findings of some previous studies in the literature [14,20].

Ki-67 is an antigen associated with cell proliferation. As it is expressed during the proliferation and synthesis phases of the cell cycle (G1, S, G2, and M), it is not expressed during G0 phase. Therefore, this antigen provides information about cells undergoing active phases of the cell cycle [8]. In the current study, we evaluated nuclear Ki-67 proliferation in order to assess proliferation. The groups were compared with regard to Ki-67 expression, and although no statistically significant difference was determined between the NGM and CAG groups ($p > 0.05$), there was a significant difference between the CAG and IM groups ($p = 0.012$).

There are conflicting reports about Ki-67 expression in patients with intestinal metaplasia and in patients with and without *H. pylori* infection.

In a study by Jung et al. [13], 20 endoscopically diagnosed cases of intestinal type gastric carcinoma, 20 cases of gastric adenoma, and 40 cases of control (normal or gastritis) were enrolled. In three groups, *H. pylori* infection rates were not significantly different. Expressions of apoptosis, Ki-67, and p53 were not significantly different in three groups.

In another study, Ki-67 expression was found to be elevated due to *H. pylori* infection, and Ki-67 expression was observed to be higher in both atrophic gastritis and intestinal metaplasia cases as compared with the patients with normal tissues [28]. Cabral et al. from Brazil found that Ki-67 expression was significantly higher in patients with *H. pylori* infection compared to those with no *H. pylori* infection. In this study, *H. pylori*-positive patients were compared with regard to CagA positivity, and Ki-67 expression was determined to be statistically significantly higher in patients positive for CagA [4].

Hoshi et al. performed a study in which they evaluated apoptosis by TUNEL assay and proliferation by Ki-67 immunohistochemical staining in gastric mucosa specimens with and without *H. pylori* infection, and no statistically significant difference was found between the two groups in terms of apoptosis and proliferation [10].

While we did not observe a statistically significant difference in terms of apoptosis in three groups, IM group demonstrated a statistically significantly higher proliferation as compared with the CAG group. Nonetheless, there was no statistically significant difference between the patients with and without *H. pylori* infection in terms of Ki-67 expression. Apoptosis and proliferation are of great importance for normal cell cycle, and the unchanged apoptosis expression despite elevated proliferation levels in our study suggests that there is no development of apoptotic response against uncontrolled cell proliferation in intestinal metaplasia patients. In the literature, many studies report that *H. pylori* infection increases proliferation [4,28]. Only the study of Hoshi et al. found no statistically significant difference between patients with and without *H. pylori* infection in terms of Ki-67 expression [10]. In our study, we determined no statistically significant difference between the patients with and without *H. pylori* infection with regard to Ki-67 expression. The statistically significantly higher Ki-67 expression in IM patients than in CAG patients indicates that there may be factors involved other than *H. pylori* in IM patients which might raise the Ki-67 expression.

Bcl-2 is a proto-oncogene and suppressor of apoptosis. Excessive production of this protein prolongs the life span of cells despite classic apoptotic stimulations [1].

In the literature, there are only a few studies focusing on Bcl-2 expression in patients with intestinal metaplasia. Anagnostopoulos et al. evaluated the Bcl-2 and Bax expression in chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia cases [1]. Bax belongs to the Bcl-2 family and is known as a pro-apoptotic protein. In this study, all the chronic gastritis cases showed Bax expression in their epithelial cells. Twenty-six percent of the atrophic gastritis cases did not exhibit Bax. Bax is further suppressed in the face of intestinal metaplasia development. Bax expression was observed only in 12% of the biopsy specimens collected from dysplasia cases. While Bcl-2 protein was not found in chronic gastritis cases, aberrant expression was observed in intestinal metaplasia and dysplasia cases. Thus, they reported suppression of Bax and overexpression of Bcl-2 during the early phase of carcinogenesis, prior to the dysplastic changes.

In Turkey, Topal et al. conducted a study and observed that *H. pylori* increased Bcl-2 expression via inducing atrophy and intestinal metaplasia in an indirect fashion. Bcl-2 positivity was higher in intestinal metaplasia than in atrophy [23].

In another study, overexpression of Bcl-2 was observed only in atrophic gastritis cases, however, it was not determined in non-atrophic intestinal metaplasia and antral gastritis associated with *H. pylori* [17].

In our study, no statistically significant difference was determined between the three groups in terms of Bcl-2 expression based on histopathological analysis. Moreover, there was no statistically significant difference between the patients with and without *H. pylori* infection with regard to Bcl-2 expression.In our study, the comparison of patients with and without a first-degree relative having gastric cancer revealed no statistically significant difference with regard to Bcl-2, Ki-67, and apoptosis ($p > 0.05$). There was no study in the literature focusing on BCL-2 and apoptosis expression in patients with a first-degree relative having gastric cancer. There are 3 studies evaluating Ki-67 expression in patients having a first-degree relative with gastric cancer. Meining et al. reported elevated antral proliferation in patients having a first-degree relative with gastric cancer, and noted increased proliferation both in the corpus and the antrum when patients with *H. pylori* infection were excluded [19]. In the other two studies, when first-degree relatives of gastric cancer patients were compared with the control group, no statistical difference was observed in terms of proliferation [22,29].

In conclusion, we observed no statistically significant difference between the IM, CAG, and NGM groups with regard to Bcl-2 expression and apoptosis, whereas we determined a statistically significantly higher Ki-67 expression in the IM group. *H. pylori* was found to elevate apoptosis, however, it had no statistically significant influence on proliferation and Bcl-2 expression. Intestinal metaplasia cases displayed no increase in apoptosis but showed elevated proliferation, which suggested that adequate apoptotic response did not occur against uncontrolled cell proliferation and that it might be an important factor in the progression from intestinal metaplasia to cancer.

In our study, we found no statistically significant difference between the patients with and without first-degree relatives suffering from gastric cancer in terms of Bcl-2 and Ki-67 expression, and apoptosis. In the literature, there are investigations studying Ki-67 expression in first-degree relatives of gastric cancer patients. However, our study was the first to evaluate apoptosis and Bcl-2 expression in patients having a first-degree relative with gastric cancer. Although this was the first study focusing on that subject in the literature, the limitation of our study was the relatively small number of patients having a first-degree relative with gastric cancer. Analyzing only 18 patients may not reflect the general population accurately. Moreover, there was no data on whether those patients had any genetic predisposing factor or not. Further studies, including larger series, should be conducted to verify these results.

Conflict of interest

None.

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