The expression of apoptosis-related proteins in patients with ulcerative colitis

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ABSTRACT

Purpose: Recent literature data indicate a key role of apoptosis in the pathogenesis of inflammatory bowel disease. The aim of the study was to evaluate the expression of Bax, Bid, Bcl-2 and Bcl-xl in non-dysplastic and dysplastic epithelium in inflamated mucosa of patients with ulcerative colitis.
Methods: The study consists of 18 patients with diagnosed ulcerative colitis. The expression of proteins was determined immunohistochemically.
Results: Lack of Bax expression in normal epithelium of the inflamed intestinal mucosa (94.4%) and a weak expression of this protein were found in dysplastic glandular cells (67%). The Bax expression of dysplastic epithelium correlates with reduced severity of chronic inflammation ($p<0.005$). Bid expression in non-dysplastic glands was found in 67% of cases vs. 16% in dysplastic epithelium that was associated with the occurrence of epithelial erosions or ulcers ($p<0.05$). Moderate cytoplasmic expression of Bcl-xl was noted in 27.7% of patients in normal epithelium and in 66.1% within dysplastic lesions. Bcl-xl expression in dysplastic glandular cells correlated with the presence of neutrophils in the lamina propria ($p <0.05$). **Conclusions:** The immunohistochemical expressions of Bax, Bcl-2 and Bcl-xl increase and Bid protein expression decreases in dysplastic glandular tubes as compared to non-dysplastic intestinal epithelium in inflamed mucosa, which may suggest an imbalance of controlled cell death in ulcerative colitis.
Key words: Ulcerative colitis, Bcl-xl, Bax, Bid, Bcl-2

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INTRODUCTION

Ulcerative colitis (UC) is a chronic intestinal multifactorial disorder of the large intestine with unknown etiology. However, the impact of genetic factors, bacterial microflora-induced infections and immune disorders has been proven [1]. UC is characterized by a widespread inflammation of the mucous membrane located in the rectum and sigmoid colon at an initial stage, subsequently involving the whole colon. Ulcers and microabscesses are also observed in the crypts, which are later replaced by dispersed connective tissue, leading to stenosis of the occupied segments in advanced cases [2].

An inflammation of the intestinal mucosa results in the disturbances of its integrity that leads to remodeling of the architecture of the intestinal villi. It occurs in the formation of intestinal epithelial defects which allow pathogens to spread into the gut mucosa. In turn, the presence of foreign antigens activates the immune cells in the lamina propria that secrete various cytokines and stimulate a natural death pathway of useless and damaged cells, i.e apoptosis [3,4]. This is an ordered, active process of gene activation by which the cell itself undergoes degradation. This process is essential during embryonic development but it also plays a role in maintaining homeostasis in adults. An excessive cell resistance to apoptosis takes part in the development of cancer or autoimmunity. There are two major ways of apoptosis: the external (membrane) as a result of membrane Fas receptor activation and the internal (mitochondrial), mainly depending on the family of Bcl-2 proteins [5]. The Bcl-2 family contains integral proteins of the mitochondrial membrane, nuclear membrane and endoplasmic reticulum that possess the pro- and anti-apoptotic properties. Pro-apoptotic proteins (Bax, Bax, Bak, Bid, Bad, Bok, and others) form between themselves homo- or heterodimers that determine the development of the mitochondrial membrane channels and release subsequent apoptosis inducers e.g. cytochrome c [6]. The activation mechanism of apoptosis is controlled by the anti-apoptotic proteins (Bcl-2, Bcl-xl) which bind to the BH3 domains of Bax and Bak, separate them and prevent their oligomerization [6].

It has been proven that apoptosis plays a significant role in the regulation of homeostasis in intestinal epithelium and controls the cellular immune response to the pathogenic agent. Increased epithelial cell apoptosis induces the loss of epithelial continuity in UC patients [7]. In addition, apoptosis also regulates stem cell population. During permanent damage, stem cells are recruited and are responsible for crypt structure rebuilding [8]. Due to the increased use of these cells for regeneration, apoptosis leads to villus atrophy and epithelial destruction [9]. The aim of this study was to evaluate the expression of Bax, Bid, Bcl-2, Bcl-xl proteins in non-dysplastic and dysplastic glandular tubes in patients with ulcerative colitis in relation to clinicopathological parameters.

MATERIALS AND METHODS

Materials

The study was performed using archive paraffin blocks of endoscopic materials obtained from 18 patients with ulcerative colitis in the years 2003- 2005. In the study group involved 7 women and 11 men, of which 55.5% patients were under 18 year old. The age of patients range from 10-74 years old (mean 35.6 ± 2 years). Disease process extends to proctitis in 6 cases, left-sided colitis in 7 cases and pancolitis in 5 cases. Sections were stained with hematoxylin and eosin (H&E) and subjected to routine histopathological assessment. According to Geboes criteria [10], the severity score and the activity of diseases were defined and graded as inactive in 1 cases, active in 15 cases and severe in 2 cases. Architectural changes, chronic inflammatory infiltrate and its composition, the destruction of intestinal crypts and the presence of erosions and ulcers were assessed too. In addition, the presence of epithelium dysplasia was noted and classified as: negative in 4 cases, indefinite in 7, low in 5 and high in 2 cases. The control group included 20 samples of the normal colonic mucous membrane of healthy individuals (10 men and 10 women) from the colon cancer screening program. The patients' age range from 34-86 years old. They did not have any evidence of acute or chronic inflammatory conditions.

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and received approval by the Local Bioethics Committee of the Medical University of Bialystok.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens were cut on a microtome into 4μm thick sections. Sections were deparaffinized in xylene and hydrated in alcohol. To block endogenous peroxidase activity, the tissue specimens were incubated with 3% H_2O_2 for 40 minutes, followed by 60 minutes in 1.5% blocking serum (normal blocking serum, goat ABC Staining System, Cat No sc-2023, Santa Cruz Biotechnology). After washing in PBS buffer (pH 7.4), the samples were incubated with the primary polyclonal antibodies: Bax (P-19, sc 526, Santa Cruz, dilution 1:100) for 95 minutes, Bid (N-19, Sc - 6539, Santa Cruz, dilution 1:100), and Bcl-xl (A-20, sC: 7122, Santa Cruz, 1:300 dilution), Bcl-2 (N-19, sC-492-G, Santa Cruz, 1:100 dilution) for 1 hour at room temperature. After the reaction LSAB technique (LSAB + System HRP, Dako, Poland), the antigen-ntibody

complex was visualized by using DAB chromogen (S3000, DAKO, Poland). Negative control section was incubated instead of the primary antibody. All section slides were counterstained with hematoxylin.

Immunohistochemical staining was evaluated by two independent pathologist blinded to the clinical information. Protein expression was determined using a semiquantitative method. The expressions of proteins observed in cytoplasm of non-dysplastic and dysplastic glandular epithelium of inflammated mucosa and were defined as a color reaction: (0)- negative (lack of expression), (+)- weak (reaction present in <30% of glandular cells), (++)- moderate (reaction present in 30-60% of glandular cells), (+++)- strong (reaction present in >60% of glandular cells) [11].

Statistical analysis

Statistical analysis was conducted based on the STATISTICA 10.0 program. Correlations between the parameters were calculated by the Pearson's correlation coefficient tests. A p-value <0.05 was considered statistically significant. The missing data was removed in pairs.

RESULTS

Histopathological examination

Histopathological analysis showed small architectural disorders in 1 case, moderate in 5 and severe diffuse or multifocal type in 12 of cases. Chronic inflammation in the degree of small, moderate and severe were found in 9, 6, 3 of patients, respectively. It also observed a moderate (4/18) and large (14/18) increase in the incidence of neutrophils in the lamina propria of biopsy materials. In the majority of cases (14/18) confirmed the presence of a significant increase in the presence of eosinophils in the lamina propria. The presence of neutrophils in the epithelium in> 50% of the occupied crypts was observed in 13 of patients. Then, the degree of intestinal crypts damage was classified. No crypt destruction was observed in 3 of cases. It noted a probable crypts destruction with local excess of neutrophils in part of these structures (2/18) and significant damage of crypts (1/18). The microscope image undoubtedly showed the destruction of the crypts in 12 of patients. It observed ulcers in 8 of cases, probable erosion in 1 and unequivocal erosion in 5 of cases. There were no similar changes in the remaining 4 of cases (Figure 1).

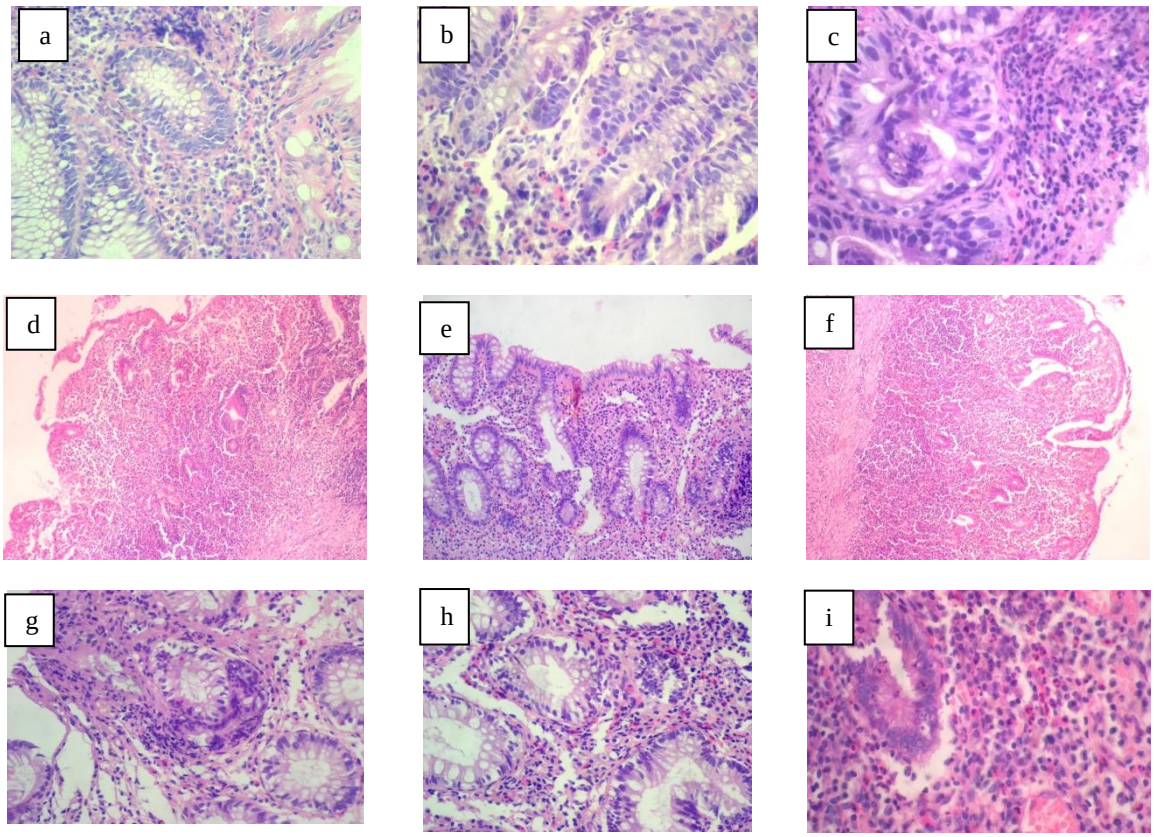

Figure 1. Histopathological characteristics of ulcerative colitis tissue (H&E). The grade of dysplasia: indefinite (a), low (b) and high (c). Colon mucosa with severe architectural changes (d) and unequivocal crypt destruction (e). Neutrophils in lamina propria and in epithelium are common for acute inflammation (f,g) while chronic inflammatory infiltrate (lymphocytes and/or plasmocytes) and eosinophils are similar to chronic diseases (h,i). Original magnification (x 100 in a, d,e,f; x 200 in g,h; x 400 in c,i).

The expression of Bax, Bid, Bcl-xl, Bcl-2 in non-dysplastic and dysplastic glandular tubes

Normal intestinal glands showed no Bax protein expression in up to 94.4% of cases, while in dysplastic tubes the expression rose to 67%. Conversely, the expression of Bid protein in non-dysplastic epithelium was observed more frequently (67%) than in the dysplastic epithelial cells (16%). However, in the case of Bcl-xl, the expression increased in the dysplastic glands (66.1%) compared to normal epithelium in inflammated mucosa (27.7%). No changes were observed in Bcl-2 protein expression. However, there were no significant differences in expression of proteins in dysplastic epithelium between grade of dysplasia (Figure 2).

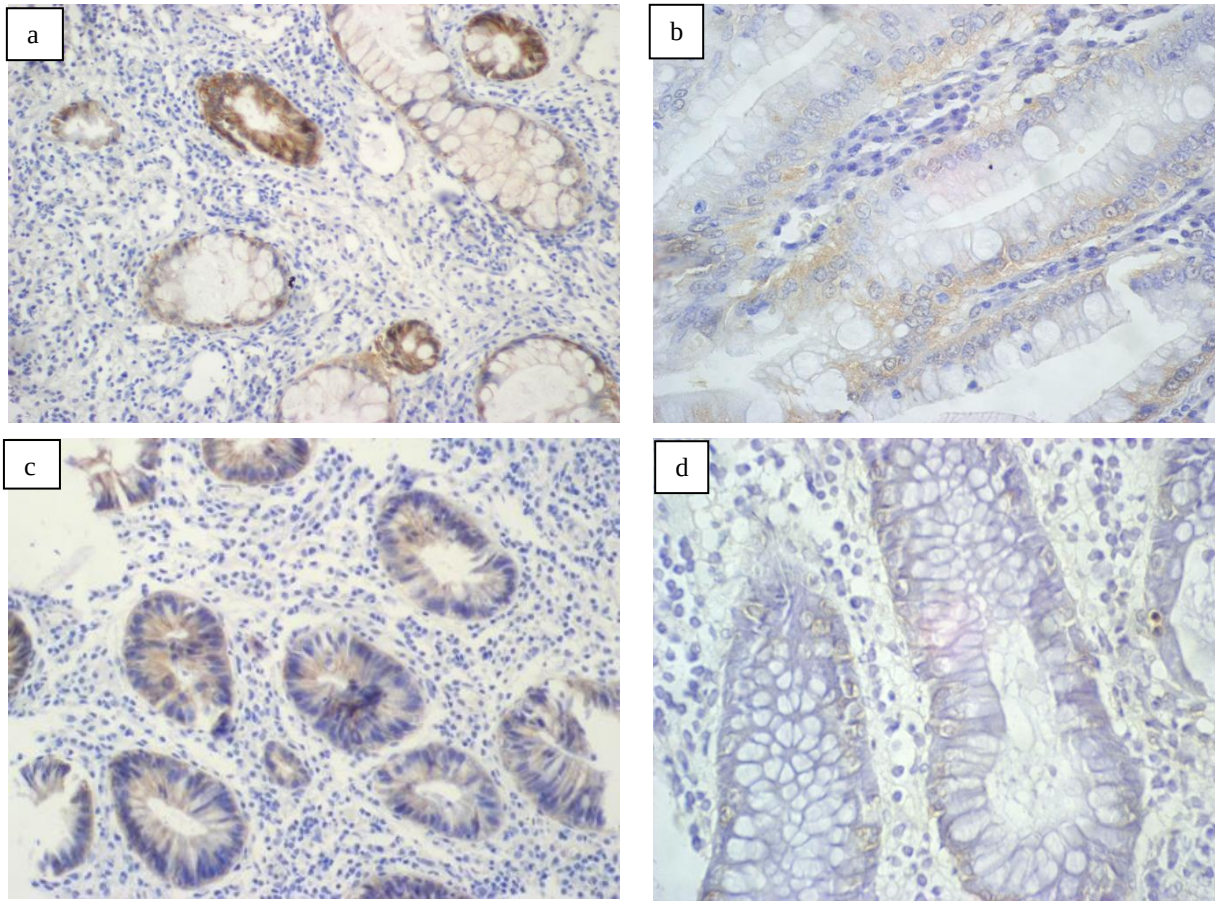

Figure 2. The expression of apoptotic proteins in non-dysplastic and dysplastic glandular tubes in inflamed mucosa (Immunohistochemistry). A positive reaction for Bax protein in dysplastic glandular tubes (a) while most of cases had a positive expression of Bid in normal epithelium of inflamed colon mucosa (b). We showed an increased in the expression of Bcl-xl in dysplastic glandular tubes (c) and a lack of reaction for Bcl-2 protein in non-dysplastic glandular epithelium (d). Original magnification (x200, 400).

The correlation between protein expressions and anatomopathological parameters

Statistical analysis revealed a negative correlation between patients' age and the Bcl-xl protein expression in normal glandular epithelium ($p < 0.05$). There were no statistically significant differences between the expression of the other proteins and such parameters as gender and disease location. However, the expression of Bax in dysplastic glandular tubes was found to positively correlate with reduced chronic inflammation infiltrate ($p < 0.005$). The positive Bid protein expression in the dysplastic epithelium was associated with the presence of erosions or ulcers ($p < 0.05$). However, the increase in neutrophils in the lamina propria correlated with increased expression of Bcl-xl protein in the dysplastic intestinal epithelium ($p < 0.05$) (Table 1).

Table 1. The correlation between anatomopathological parameters and expression of proteins in UC patients.

Parameter		**No. of cases (%)**	**Expression of proteins in non-dysplastic and dysplastic glandular tubes***			
			Bax	**Bid**	**Bcl-2**	**Bcl-xl**
Age	≤18	11 (61.1%)	NS	NS	NS	***p*<0.05**
	>18	7 (38.9%)				
Gender	Male	11 (61.1%)	NS	NS	NS	NS
	Female	7 (38.9%)				
Localization/ Extent of disease	Proctitis	6 (33.3%)	NS	NS	NS	NS
	Left-sided colitis	7 (38.9%)				
	Pancolitis	5 (27.7%)				
Histopathological findings						
Grade of dysplasia	Negative	4 (22.4%)	NS	NS	NS	NS
	Indefinite	7 (38.9%)				
	Low	5 (27.7%)				
	High	2 (11.1%)				
Grade of inflammation	Inactive	1 (5.7%)	NS	NS	NS	NS
	Active	2 (11.1%)				
	Severe	15 (83.2%)				
Architectural changes	Absent	0 (0%)	NS	NS	NS	NS
	Mild	1 (5.7%)				
	Moderate	5 (27.7%)				
	Severe	12 (66.6%)				
Chronic inflammatory infiltrate	Absent	0 (0%)	***p*< 0.005**	NS	NS	NS
	Mild increase	9 (50%)				
	Moderate increase	6 (33.3%)				
	Marked increase	3 (16.6%)				
Lamina propria neutrophils	Absent	0 (0%)	NS	NS	NS	***p*<0.05**
	Mild increase	0 (0%)				
	Moderate increase	4 (21%)				
	Marked increase	14 (79%)				
Lamina propria eozynophils	Absent	0 (0%)	NS	NS	NS	NS
	Mild increase	0 (0%)				
	Moderate increase	4 (21%)				
	Marked increase	14 (79%)				
Neutrophils in epithelium	Absent	0 (0%)	NS	NS	NS	NS
	<5% crypts involved	0 (0%)				
	<50% crypts involved	5 (27.8%)				
	>50% crypts involved	13 (72.2%)				
Crypts destruction	Absent	3 (16.6%)	NS	NS	NS	NS
	Probable- local excess of neutrophils	2 (11.1%)				
	Probable- marked attenuation	1 (5.7%)				
	Unequivocal crypt destruction	12 (66.6%)				
Erosion/ Ulceration/ Granulation tissue	Absent	4 (21%)	NS	***p*<0.05**	NS	NS
	Recovering epithelium	0 (0%)				
	Probable erosion	1 (5.7%)				
	Unequivocal erosion	5 (27.8%)				
	Ulcer or granulation tissue	8 (45.5%)				

Pearson's correlation coefficient test. Missing data were removed in pairs. NS- non significant

DISCUSSION

Apoptosis, known as a programmed cell death, can occur in cellular organelles-associated way (mitochondrial pathway) that involves the Bcl-2 protein family. These proteins belong to a group of oncogene products that regulate the process of programmed cell death [5]. Literature reports indicate the presence of apoptosis in crypts and luminal surface in normal intestinal epithelium [12]. Our research confirms the participation of pro- and anti-apoptotic proteins in both normal and dysplastic epithelium of patients with UC. Only a limitation of our study is small group, however, the detailed analysis allowed to compare our results with the literature data.

We noted absent or weak cytoplasmic expression of Bax protein in normal glandular epithelium of the inflamed colonic mucosa in 94.4% and 5.6% of UC cases, respectively. These observations were in line with Limura et al. [13], who evaluated the expression of apoptotic proteins in the inflamed colonic mucosa during ulcerative colitis. Bax protein expression was weak in epithelial cells on the luminal surface and in epithelial cells of the whole crypts in active UC. Also Karamanolis et al. [14] assessed the role of Bcl-2/Bax system in patients with ulcerative colitis. The authors demonstrated a decrease in Bax protein expression in intestinal epithelial cells in patients with moderate or severe clinical course of the disease but still significantly higher than in healthy controls. It is suggested that the decrease in Bax expression may be associated with a local inflammatory process [13]. In opposition to our results, Kouklakis et al. [15] reported the overexpression of Bax in colonic biopsy specimens of 50% UC patients. Besides, Leal et al. [16] found a strong Bax expression in the UC group confirmed by a high level of this protein in ileal pouches using Western Blot analysis. Patients with long-standing UC have an increased risk of lesions that predispose to carcinogenesis. The detection of intestinal epithelial dysplasia, often multifocal, as well as defining its level (low / high grade) can be crucial for the prevention of tumor development from these lesions. Therefore, we provided analysis of dysplastic glandular epithelium in which we observed a positive expression of Bax protein in more than half of the cases compared to sporadic reaction of this protein in normal epithelium. Our findings have been confirmed by Yoshida et al. [17], who observed stronger Bax protein expression in both upper and lower crypts in patients with low-grade dysplasia and high-grade dysplasia as compared to the control. In addition, the reaction of the protein in patients with UC-associated invasive carcinoma was stronger than in normal or regenerating glandular epithelium of the crypts. These observations allow us to argue that the increase in Bax protein expression in the dysplastic glandular cells may indicate an increased apoptotic activity of these cells which may lead to the development of advanced and malignant cancerous lesions. Furthermore, our studies revealed a correlation between the increase in Bax protein expression in the dysplastic glands and reduction in chronic inflammation infiltrate. It is well known that chronic inflammation is a persistent exponent of the disease process and the cells involved undergo substantial usage. It is also believed that the deficiency/dysfunction of the cellular immune response in an ongoing inflammatory process probably stimulates other mechanisms that regulate homeostasis of glandular cells, including apoptosis. Similar observations have been noted by Seidelin et al. [18], who described association between the degree of local inflammation and an increase in intestinal epithelial cell apoptosis.

We also performed immunohistochemical analysis of pro-apoptotic Bid protein. We observed a weak expression of this protein in non-dysplastic epithelium in comparison to the dysplastic lesions in which only a few cases showed a positive response. Furthermore, statistical analysis revealed that the positive expression of Bid in intestinal epithelium dysplasia was correlated with the presence of epithelial erosions or ulcers. Due to the lack of reports on Bid protein expression, the role of this protein cannot be clearly identified in the pathogenesis of UC. However, our results suggest that a sporadically positive protein expression found in the dysplastic glandular epithelium indicates inhibition of autodestruction of these cells.

Next, our findings showed mainly a weak expression of Bcl-xl protein in the cytoplasm of normal intestinal epithelium in most cases, which was associated with age. In contrast to the normal epithelium, the presence of moderate protein expression was demonstrated in a large percentage of lesions located in dysplastic epithelium. It seems that this protein plays an essential role in determining the mechanisms of excessive resistance to cell apoptosis. Our results are partly in conformity with the findings of Van der Woude et al. [19], who evaluated the expression of apoptotic proteins, including Bcl-xl in patients with UC-associated dysplasia and neoplasm. Those authors showed a lack of protein reaction in patients with chronic UC. However, there were weak and moderate expressions in the accompanying dysplastic changes, and a strong reaction in tumor cells. In addition, statistical analysis of our results showed a correlation between the increase in neutrophils in the lamina propria and stronger expression of Bcl-xl in the dysplastic intestinal epithelium. A significant number of neutrophils seen in the crypts and within the epithelium accompanied an early or acute disease stage.

Subsequently, we saw abscesses in the crypts and first signs of crypt destruction. An excess of these cells in the lamina propria may modify the apoptotic properties and determine the elevated defense mechanism of intestinal epithelium dysplasia. It is therefore recommended to minimize the inflammation of the intestine in UC patients to prevent the development of cancer [19].

Moreover, we did not see the expression of anti-apoptotic Bcl-2 protein in any of the structures in the majority of patients (83%), but only individual cases showed a weak color reaction of the protein. Our observations have been confirmed by Bruwer et al. [20]. The authors demonstrated the presence of weak expression of Bcl-2 protein in a few cases with low-grade and high-grade dysplasia, and much stronger in patients with colorectal carcinoma. Limura et al. [13] observed a strong reaction of Bcl-2 in basal surface of the crypts that weakened along with locating upwards epithelium (luminal surface) in active colitis and normal colonic epithelium. Mikami et al. [21] also found an increase in the expression of Bcl-2 protein at the lower halves of the crypts compared to the upper halves of the normal, regenerating, low- and high-degree dysplastic epithelium. Other reports have also confirmed the overexpression of Bcl-2 in patients with UC [15-16,22,23]. The multiplicity of all published reports hinders explicit explanation of the function of Bcl-2 protein. However, our findings point to the fact that the protein is probably not involved in the programmed death process of normal and dysplastic glandular cells in patients with UC.

In conclusion, the immunohistochemical expressions of Bax, Bcl-2 and Bcl-xl proteins increase and Bid protein expression decreases in dysplastic glandular tubes as compared to non-dysplastic intestinal epithelium in inflamed mucosa, which may suggest an imbalance of controlled cell death in ulcerative colitis.

Conflicts of interests

The author declared that they had no conflicts of interest with respect to the authorship and/or publication of this article**.**

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