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COX-2 and Ki-67 immunohistochemical markers in the assessment of long-standing ulcerative colitis associated dysplasia

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Abstract

Introduction: Malignancy in LUC (long-standing ulcerative colitis) presumably evolves through a chronic inflammation–dysplasia–adenocarcinoma sequence in which a multitude of yet not fully understood factors takes part. *Aim*: To assess ulcerative colitis (UC) associated dysplasia and to distinguish regenerative changes from premalignant ones using immunohistochemical (IHC) markers. *Materials and Methods*: We studied 80 LUC biopsy specimens: 20 high-grade dysplasia (HGD), 20 low-grade dysplasia (LGD), 20 indefinite for dysplasia, 20 regenerative atypia. We used anti-COX-2 and Ki-67 antisera (Dako, Carpinteria, USA) to perform immunohistochemical staining by the labeled Streptavidin–Biotin method, and then assessed and graded staining intensity and distribution using previously described scoring systems. Statistical analysis was made using *chi*-square test and SPSS application. A *p*-value <0.05 was considered significant. *Results*: In LGD, most of the cases had middle and top Ki-67 localization of the staining. For HGD, we found to be characteristic the top and surface staining of the crypts and no case of basal immunostaining. COX-2 immunostaining was positive (total score ≥3) in 72.5% of all the UC cases studied. In non-dysplastic lesions (regenerative atypia), COX-2 expression was negative and as the pathologic process progressed towards dysplasia/malignant transformation, COX-2 expression became positive with a progressive increase of the total score. *Conclusions*: A combination of enhanced colonoscopic surveillance and IHC markers those are more sensitive for dysplasia might be the optimal way to manage the increased colorectal cancer (CRC) risk in LUC patients. Further studies to find additional prognostic parameters will provide valuable insights into the behavior of LUC.

Keywords: ulcerative colitis, dysplasia, colorectal cancer, Ki-67, COX-2.

Introduction

As stated by the *European Crohn and Colitis Organization* (ECCO), ulcerative colitis (UC) is a chronic inflammatory disease that affects the colonic mucosa [1]. Unfortunately, its global incidence remained relatively stable in the last three decades [2, 3]. In Europe, the existence of a North–South gradient was observed, with an increasing incidence of the disease in the developing countries [4–6].

In the same time, UC is the only inflammatory disease in which a high incidence of carcinogenesis was reported, especially in patients with long-standing disease (>7–10 years). This suggests that the prolonged inflammation of the mucosa or the repetitive regenerative processes lead to epithelial changes that either initiate or predispose to dysplasia.

Malignancy in UC presumably evolves through a chronic inflammation–dysplasia–adenocarcinoma sequence in which a multitude of yet not fully understood factors takes part. Dysplasia often arises multifocal and is difficult to be recognized by colonoscopy [7]. It is also challenging to differentiate dysplasia from regenerative changes.

The grade of dysplasia is important as it has a great impact upon the sensitivity and specificity of the presence and further development of colorectal cancer. Dysplasia, irrespective of its grade, was reported to have 74% sensitivity in the development of cancer [8]. In the most recent meta-analysis, low-grade dysplasia was associated with a 9-fold increase in developing colorectal cancer and a 12-fold increase in developing advanced neoplasia [9].

Routine histological evaluation (Hematoxylin–Eosin) is still a standard method in grading dysplasia and assessing regenerative epithelial changes. The histological grading of dysplasia (low-grade to high-grade) has limitations such as interobserver variability, confounding inflammatory changes, and particularly the random nature of biopsies [10]. Therefore, we need additional, affordable methods to increase the accuracy and precocity of the diagnosis.

The aim of our work was to find additional methods to assess UC associated dysplasia, to distinguish regenerative changes from premalignant ones, in order to increase the accuracy and precocity of dysplasia diagnosis. Our morphopathological and immunohistochemical study also tried to find possible correlations between COX-2 expression and Ki-67 as well as particular aspects related to the distribution and intensity of the immunostaining.

Materials and Methods

For our retrospective study, we chose, with the help of two independent pathologists, the cases of 80 patients

(aged 53.5±14.2 years, 42 women and 38 men), with previously established diagnosis of LUC, who addressed the Ist Internal Medicine Clinic of the Emergency County Hospital of Oradea, Romania, during the years 2006–2010. All these patients were hospitalized with suspected inflammatory bowel disease, clinically and endoscopically investigated. The colonoscopy was performed with an Olympus Exera CLE145 video-endoscope in the Endoscopy Unit of the same Clinic.

Biopsies taken during colonoscopy were fixed in 10% formaldehyde for a period of 24–48 hours, included in paraffin and sectioned at 3–4 μm. There have been multiple sections, serial (average 8–10/case). For more accurate pathological diagnosis and inclusion into a group lesion, the first sections were stained with the usual histological methods. Routine morphological examination was performed on sections stained with Hematoxylin–Eosin, standard technique, applied to all studied cases. Microscopic examination of biopsies allowed the grouping and classification of lesions.

The patients were divided into four groups, depending on the type of histological lesion: 20 cases with high-grade dysplasia (HGD), 20 cases with low-grade dysplasia (LGD), 20 cases with indefinite for dysplasia and 20 cases with regenerative atypia (Figure 1), according to the criteria of the *Study Group on Inflammatory Bowel Disease and Morphology of Dysplasia* and *Vienna classification* [11]. This histological group selection was done for an initial, preliminary assessment of dysplastic changes, which were later confronted with the immunohistochemical results for accuracy and precocity purposes.

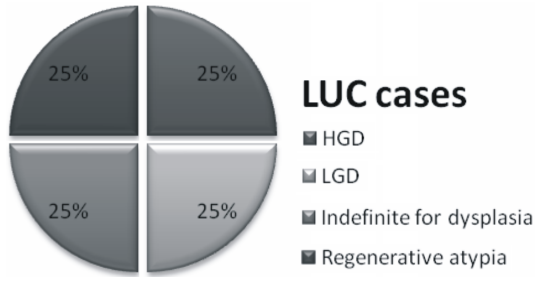

Figure 1 – ***Distribution of lesions in our patients.***

The immunohistochemical technique used in our study was the LSAB–HRP system (labeled Streptavidin–Biotin), supplied by Dako, Carpinteria, USA. The method is based on the application of biotinylated secondary antibody and alkaline phosphatase or peroxidase-labeled Streptavidin. The technique provides intense immunostaining using primary antibodies highly diluted in Tris-HCl buffer, pH 7.6. This reaction was applied for following antibodies: anti-Ki-67, clone MIB-1 and anti-COX-2, clone CX-294 (Dako, Glostrup, Denmark).

The time steps of the LSAB-2 Kit/HRP technique comprised: dewaxing and rehydration of the sections; pretreatment by boiling the sections in citrate buffer, pH 6, for 30 minutes at 80^{0}C; blocking endogenous peroxidase with hydrogen peroxide for 5 minutes; incubation with diluted primary antibody for 10–30 minutes; incubation with biotinylated secondary antibody for 10 minutes; incubation with Streptavidin-peroxidase for 10 minutes; incubation with extemporaneously prepared chromogen (3,3'-diaminobenzidine – DAB) for 10–20 minutes; counterstained with Hematoxylin for 2–3 minutes; installation in Eukitt (DAB).

The proliferative capacity of the investigated lesions was tested immunohistochemically using anti-Ki-67 antibody. MIB-1 monoclonal antibody recognizes the cell proliferation nuclear antigen (a protein doublet with molecular weights of 345 and 395 kDa) expressed in all phases of the cell cycle except G0 phase. We used the same immunohistochemical technique for the diluted MIB-1 antibody, the sections being pretreated by boiling for 60 minutes at 90^{0}C in retrieval solution. The final product has a brown reaction (visualizing agent – DAB) and sometimes nuclear and cytoplasm localization. For the positive control reaction, we included in the study a fragment of tonsil, and for the negative control, the buffer replaced the primary antibody.

The immunohistochemical expression of COX-2 protein was tested using anti COX-2 mouse monoclonal antibody (clone CX-294, Dako, Denmark), with the primary antibody, in a 1:50 dilution, and then applying the secondary antibody for 30 minutes. Positive cellular staining pattern was cytoplasmatic, with a brown reaction (visualizing agent – DAB) and a light purple for background.

Positive control was performed using colonic adeno-carcinoma. Negative control was performed by replacing primary antibodies with the buffer.

The Ki-67 reaction was considered positive for any nuclear staining, regardless of the intensity of the reaction. Cells were considered Ki-67 positive in the presence of brown granular or diffuse nuclear staining. Cells in mitosis had a nuclear and cytoplasmic staining associated.

Quantification of the reaction was performed by assessing a Ki-67 index marker (Ki-67 IM), expressed as a percentage of the Ki-67 positive tumor cells reported to 500 positive and negative Ki-67 tumor cells.

The evaluation of Ki-67 immunoassaying was carried out dividing the cases into four groups depending on the extent of Ki-67 staining:

- 'basal zone': staining restricted to the basal third of the crypt;
- 'mid zone': extension of staining into the middle third;
- 'top zone': extension into the upper third;
- 'surface': extension into the surface epithelium.

For COX-2 evaluation, we used a previously described scoring system with staining intensity graded as: "1" – weak, "2" – moderate or "3" – strong, and the positively stained area as "0" (not stained), "1" (focal staining of <1/3 of the cells), "2" (staining of <2/3 of the cells), and "3" (majority of the cells are stained) [12]. Total scores (grade and area) of ≥3 were defined as positive and those <3 as negative.

Positive Ki-67 and COX-2 staining was brown and the background staining was light purple/blue. Two observers assessed all sections blindly.

Statistical analysis

For the inference of our results, *chi*-square test was used to analyze the relationship between the staining intensity and distribution of Ki-67 and COX-2 in association with different types of lesions. A *p*-value <0.05 was considered significant. Statistical analysis was performed with SPSS application.

Results

The analyzed dysplastic specimens were diagnosed mainly in patients aged 53.8±8.8 for LGD and 67.8±6.9, for HGD, having more than 12 year's duration of the disease, extensive localization and moderate endoscopic forms (Table 1).

Table 1 – ***Epidemiological and histopathological parameters***

Women/men		38/42	LGD	HGD
Age [years]	*Medium*	53.5±14.2	53.8±8.8	67.8±6.9
Disease duration	*5–10 years*	48.75% (39 cases)	8	4
	>10 years	51.25% (41 cases)	12	16
Extension	*Proctitis*	12.5 % (10 cases)	1	0
	Left colon	55% (44 cases)	12	6
	Extensive/ Pancolitis	32.5% (26 cases)	7	14
Endoscopic index	*Mild*	35% (28 cases)	2	0
	Moderate	43.8% (35 cases)	11	11
	Severe	21.2% (17 cases)	7	9

HGD – High-grade dysplasia; LGD – Low-grade dysplasia.

Ki-67 immunostaining

We observed that in regenerative atypia cases, there is a predominance of restricted Ki-67 staining to the basal third of the crypt (50%) which appears to exclude a diagnosis of dysplasia (Figure 2). Surface staining was noted in only two regenerative atypia cases, associated with massive polymorphonuclear inflammatory infiltrate and with the existence of superficial erosions. We noticed the extension of the proliferation compartment in eight cases of ulcerative colitis, in four (20%) cases in the middle third of the glandular crypt and in four (20%) cases in their upper zone (Table 2).

In indefinite for dysplasia cases of UC, we noticed a slight increase of proliferation activity in the upper part of the glands, three cases having top staining and six cases having "surface" staining. The presence of ulcerative colitis associated dysplasia showed the obvious extension of the proliferative compartment of the glandular epithelium. Thus, in LGD (Figure 3), we met only two (10%) cases with basal immunostaining, eight (40%) cases with middle glandular staining, seven (35%) cases with top positive staining, and three (15%) cases with positive reactions along the surface epithelium. In HGD, we have not identified any case with Ki-67 positive staining restricted in the basal glandular zone. The Ki-67 staining extended to the top and surface area was characteristic to severe dysplasia (80%) (Figure 4).

In LGD, most of the cases had middle and top Ki-67 localization of the staining. For HGD, we found to be characteristic the top and surface staining of the crypts and no case of basal immunostaining.

COX-2 staining distribution

In our study, we observed that COX-2 distribution within individual colonic crypts was diffuse, revealing no consistent localization to crypt base, mid-crypt, or surface. The expression of COX-2 in dysplastic lesions (LGD, HGD) was present in most (>90%) of the cells (Figures 5 and 6). The staining intensity showed moderate to strong cytoplasmic overexpression in all of the dysplastic biopsies, regardless of grade (Table 3).

Table 2 – ***Interpretation of Ki-67 immunohistochemical reactions***

Ki-67 extension	Regenerative atypia (20 cases)	Indefinite for dysplasia (20 cases)	LGD (20 cases)	HGD (20 cases)	Statistical analysis	
Basal zone	10	3	2	0	0.002	*p*
Middle zone	4	8	8	4	9	*df*
Top zone	4	3	7	7	26.229	χ^2
Surface	2	6	3	9		

HGD – High-grade dysplasia; LGD – Low-grade dysplasia.

Table 3 – ***Interpretation of COX-2 immunohistochemical reactions***

COX-2 staining		Regenerative atypia (20 cases)	Indefinite for dysplasia (20 cases)	LGD (20 cases)	HGD (20 cases)	Statistical analysis	
Negative (<3)		20	17	1	0	0.002	*p*
Positive	*3*	0	1	1	0	9	*df*
	4	0	2	18	0	203.135	χ^2
	≥5	0	0	0	20		

HGD – High-grade dysplasia; LGD – Low-grade dysplasia.

In non-dysplastic lesions (regenerative atypia), COX-2 expression was negative (Figure 7) and as the pathologic process progressed towards dysplasia/ malignant transformation, COX-2 expression became positive with a progressive increase of the total score. Therefore, we identified 42 COX-2 positive specimens (total score ≥3, 52.5% from the 80 patients studied). Difficult cases, interpreted like indefinite for dysplasia, were negative in 85% of all from this category.

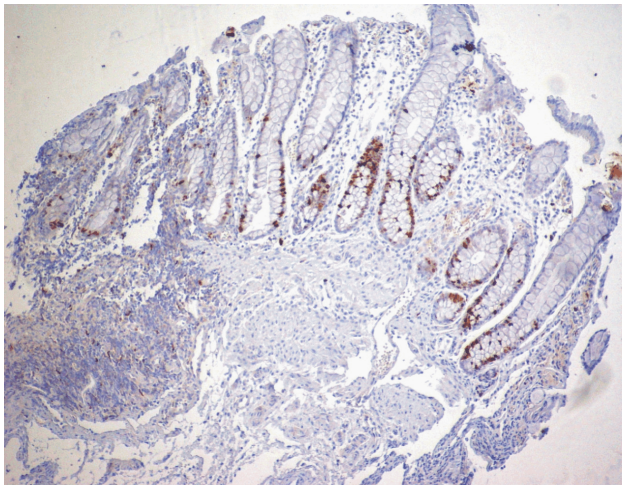

Figure 2 – ***Restricted Ki-67 staining to the lower 1/3 of the glandular crypt, ob. 10×.***

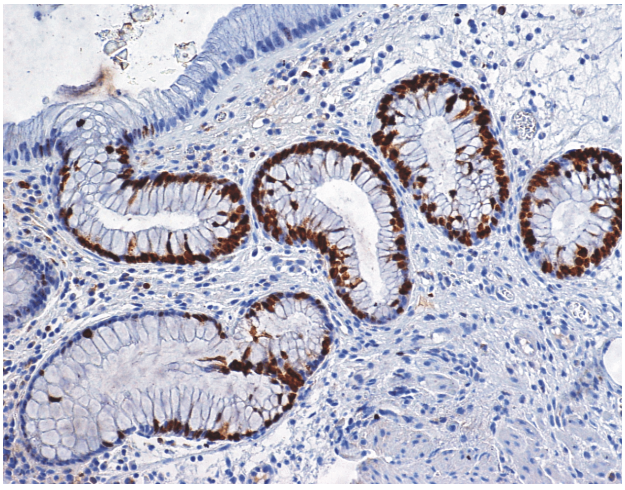

Figure 3 – ***Ki-67: extension of the proliferative compartment to the top of an atrophic gland, ob. 20×.***

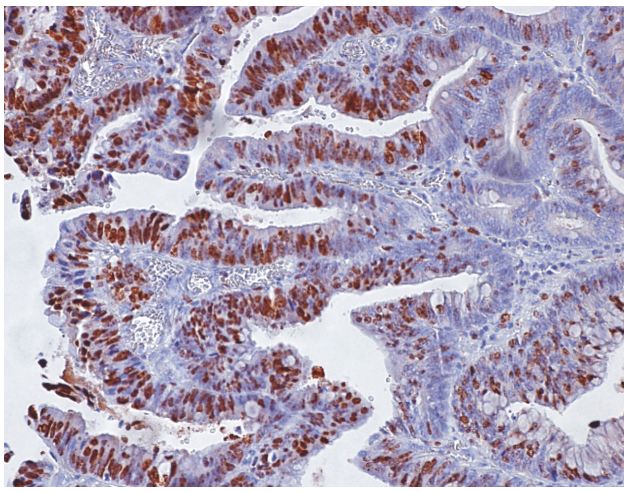

Figure 4 – ***HGD: Ki-67 surface staining, ob. 20×.***

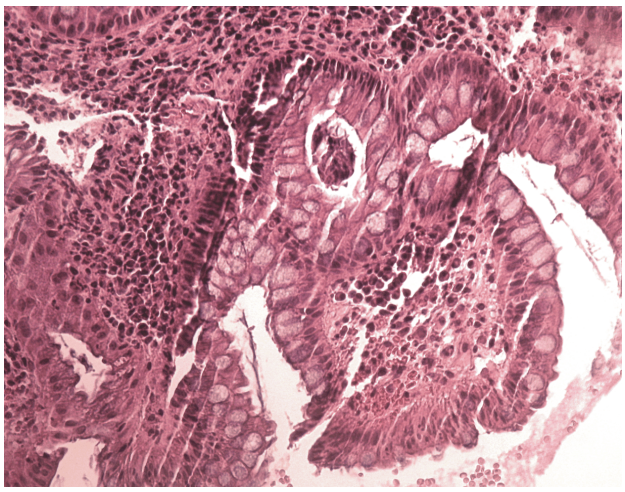

Figure 5 – ***LGD: COX-2 positive (total score 3), ob. 20×.***

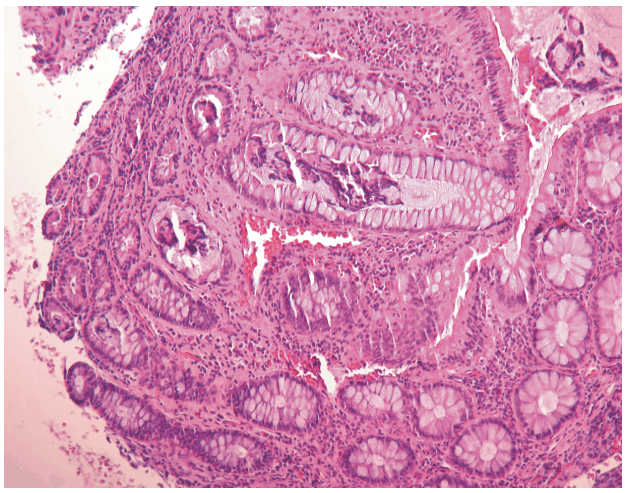

Figure 6 – ***HGD: COX-2 positive (total score 5), ob. 10×.***

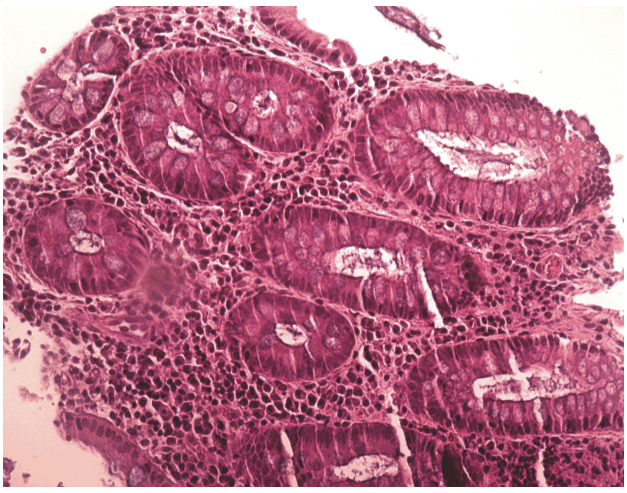

Figure 7 – ***COX-2 negative (total score 1), ob. 20×.***

COX-2 and Ki-67 immunostaining correlations

COX-2 negative staining was correlated with Ki-67 restriction into the basal third of the crypt suggesting non-dysplastic lesions. COX-2 expression became positive with a progressive increase of the total score and correlated with diffuse Ki-67 staining as the pathologic process progressed towards dysplasia/ malignant transformation. Three indefinite for dysplasia UC cases with surface Ki-67 staining, proved to be COX-2 positive. These results allowed us to readmit them into the UC associated dysplasia cases (Table 4).

Table 4 – ***Correlations of Ki-67 and COX-2 immunohistochemical reactions***

		COX-2				Statistical analysis	
		Negative	*Positive 3*	*Positive 4*	*Positive 5*		
Ki-67	*Basal*	15	0	0	0	0.001	*p*
	Middle	11	1	8	4	9	*df*
	Top	6	1	7	7	29.177	χ^2
	Surface	6	0	5	9		

Discussion

Because dysplastic changes of the colonic mucosa are associated with an increased risk of colorectal cancer in UC, it is necessary to have a colonoscopy-screening program with the aim of decreasing mortality and morbidity secondary to colorectal cancer and at the same time to avoid unnecessary prophylactic colectomies [13, 14]. Taking into account this premises stated by previous published papers, we initiated our study in an attempt to find additional methods to detect high-risk cases in due time and to make screening programs more accurate. We also wanted to find an affordable technique to assess dysplasia, as the actual economical state in many countries restricts the access to PCR techniques. It is obvious that by having an early diagnosis of the exact type of dysplasia the access to a proper treatment is granted in due time, although our study did not evaluate the mortality and morbidity associated with our results.

In 1974, Lipkin M, and Deschner EE later in 1982, found abnormal proliferative cellular lesions in colonic crypts in humans and animals with familial adenomatous polyposis, sporadic adenomas and carcinomas, and in ulcerative colitis. The two researchers showed that increased DNA synthesis might be a step in a final common pathway leading to malignant transformation [15, 16]. Also, Deschner EE *et al.* published a paper focused on ulcerative colitis in which they observed that in normal colonic mucosa, the predominant area of cell proliferation is localized to the lower one third of the crypts; cells then migrate from the base of the crypt upwards to the luminal surface, where they are sloughed off [17].

Therefore, our results, which show that Ki-67 top and surface immunostaining is an important clue of UC associated dysplasia, have a previously demonstrated physiopathological real reason. Also, there are several published papers [18, 19] that analyze the characteristics of proliferative compartments in adenomas which appear to expand to the epithelial surface. For HGD, we found to be characteristic the top and surface staining of the crypts and no basal immunostaining.

Noffsinger AE *et al.* explained the existence of a different Ki-67 staining pattern in dysplastic lesions and carcinomas compared to regenerative epithelium, with positive cells often confined to the top of the crypts, which might help to delineate areas of dysplasia where distinction from regeneration based on histopathology alone is difficult [20]. Our study showed that restriction of Ki-67 to the basal third of the crypts is not a characteristic of epithelial dysplasia. Also, the restriction of Ki-67 staining to the 2/3 of the crypt excluded the diagnosis of HGD. Moreover, malignant transformation is also associated with a disruption of the normal structural organization of the growth compartment, because most high-grade dysplasia had a diffuse staining pattern with scattered Ki-67 positive cells.

COX-2 was shown to be responsible for the increase in COX activity in inflammation; increased concentrations of COX-2 have been found in synovial tissue during attacks of rheumatoid arthritis [21].

The immunoreactivity of COX-2 was observed in epithelial and inflammatory cells in 69% of patients with UC, indicating that this immunoreactivity is strongly induced not only in *lamina propria* cells but also in apical epithelial cells of the colonic mucosa in IBD [22, 23].

COX-2 immunostaining was according to our work, very intense in HGD and the intensity of the staining decreased when the severity of glandular dysplasia diminished. The increased COX-2 staining in buffer areas compared to normal mucosa and UC mucosa might be explained by the appearance of borderline dysplastic changes.

COX-2 immunostaining revealed in our study chromogenic variations ranging from moderate to marked staining in dysplasia cases. The variation was proportional with the dysplasia grade. Most of the dysplastic cells showed COX-2 positive staining.

Singer II *et al.* [23] studied in their work the expression of COX-1 and COX-2 in UC cases, Crohn's disease and normal tissue, and showed that there was a greater immunolabeling of COX-2 in epithelial cells of UC and Crohn's disease samples. These findings suggested that the persistent expression of this protein might be related to an increased risk of carcinogenesis.

The exact analysis of COX-2 in the malignant transformation pattern of the UC mucosa might be the solution of new therapeutically horizons. Our results, as well as their correlation with other international studies, could not offer the possibility of the exclusive use of COX-2 staining in the evaluation of predictability. There was neither consistent areal pattern of the COX-2 immunostaining (base crypt, middle zone or top zone) nor an intensity pattern. The analysis of transitional mucosa (LGD) reveals a maximum score of 4. Scores ranging from 4 to 5 or more assures a suggestive profile for future malignant tumors.

From our knowledge, there is only one study that combines Ki-67 with COX-2 immunostaining but it was made on colorectal cancer cases and it also associated p53 staining [24]. However, in this study, three colorectal cancer cases were associated with UC, but "they were not stained intensely" with COX-2. They also mentioned that the three UC associated colorectal cancers, showed intense p53 staining in limited areas, but no COX-2 or Ki-67 staining. Also, there were no statistically relevant correlations between COX-2 and Ki-67 expression in the aforementioned study, probably due to the rather small (only three) number of studied cases.

We found that the HGD cases were COX-2 intensely positive (≥5). Due to our observation that COX-2 expression had a progressive increase of the total score

as the pathologic process progressed towards dysplasia/ malignant transformation, it is assumable that if we would have had a group of confirmed adenocarcinomas, the COX-2 staining would show positive expression like in HGD. Also, the statistical analysis performed on our 80 cases showed a correlation between COX-2 staining Ki-67 staining in advanced dysplasia cases (χ^2=29.177, *df*=9, *p*=0.001).

The inconsequentiality of intensity and localization of COX-2 staining reveals the necessity of using an additional marker in the evaluation of UC evolution.

In our study, based on a combined statistically relevant analysis of the two IHC markers we obtained a better relevance than the separate analysis of Ki-67 or COX-2 alone. Adding these imunohistochemical markers to routine histological assessment might improve the accuracy of early detection of precancerous stages in long-standing ulcerative colitis patients.

Conclusions and future perspectives

In our experience, UC associated dysplasia developed in people with moderate endoscopic forms of the disease but with extensive localization and long duration of the disease. Ki-67 immunoexpression can increase the accuracy of dysplasia diagnosis and together with COX-2 marker could be combined with routine histological evaluation in long-standing UC to improve the diagnostic yields especially for identifying patients with high risk of malignant transformation, who might benefit of early and proper treatment. A combination of enhanced colonoscopy surveillance and IHC markers that is more sensitive for dysplasia might be the optimal way to manage the increased CRC risk in these patients. Further studies to find additional prognostic parameters will provide valuable insights into the behavior of LUC.

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