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The value of histological changes and immunohistochemical markers Ki67 and p53 in the assessment of ulcerative colitis related dysplasia

Research Article

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Abstract: The risk of carcinoma increases in patients with a 10-year or longer duration of ulcerative colitis (UC). To search for a more objective parameter to assess epithelial dysplasia. The study comprised 25 cases of longstanding UC: 7 cases with regenerative atypia, 7 with low grade dysplasia, 7 with high grade dysplasia, and 4 cases indefinite for dysplasia. The colonic biopsies obtained during endoscopy were stained with H&E to identify the aforementioned categories. Seventy-five sections from biopsy specimens were stained immunohistochemically to detect differences in the frequency and pattern of nuclei positive for the proliferation marker Ki67 and p53. In high grade dysplasia, the distribution of Ki67 positive cells was diffuse throughout the full length of the crypt, whereas low grade dysplasia and epithelium indefinite for dysplasia, as well as regenerative epithelium, showed an expanded basal zone. None of the regenerative atypia cases showed strong intensity p53 staining compared to dysplasia cases. None of the high grade dysplasia cases showed restricted p53 staining to the lower two thirds of the crypt. All the cases of HGD showed extension of Ki67 and p53 staining above the basal two thirds of the crypt. Ki67 and p53 immunostained cell assessment combined with routine histological evaluation of colorectal mucosa can improve the diagnostic accuracy, as well as the assessment of malignant transformation risk.

Keywords: *Ulcerative colitis • Histopathology • immunohistochemistry • Chronic inflammation • Dysplasia • Carcinoma*

1. Introduction

Ulcerative colitis (UC) is commonly characterized by continuous inflammation or cyclic episodes of relapse and remission. On the histological level, the cycling or ongoing disease activity is accompanied both by increased numbers of apoptotic cells throughout the crypt axis and by corresponding epithelial lining of regenerating areas [1].

Colorectal carcinoma is a dreadful complication of UC. The risk of developing cancer increases in patients with longstanding UC (7 to 10 years) with a rate of approximately 0.5-1% per year and therefore surveillance colonoscopy has been widely recommended [2]. In most instances, cancer evolves through a neoplastic process called dysplasia. Dysplasia (intraepithelial neoplasia) is present in more than 70% of patients with chronic UC and cancer, coinciding with the location of the cancer, which arises from chronically inflamed mucosa [3].

Dysplastic lesions are difficult to recognize by means of endoscopy. It is also a tough task to differentiate them histologically from inflammatory regenerative changes of the epithelium. The efficacy of current surveillance remains unsatisfactory and thus, it becomes clear that we need additional methods to improve the early detection of cancer.

Evaluation of dysplasia in long-standing UC is a difficult and often subjective task. Although the routine pathological examination provides important information

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about the chronic inflammation-carcinoma sequence, the relationship between the morphological changes or remodeling of regenerated mucosa, and carcinoma development, is yet to be clarified.

In order to overcome these difficulties, adjunctive modalities for diagnosing UC-associated neoplasia, chromo- and magnifying endoscopy for endoscopic diagnosis, and analysis of p53 and Ki67 alteration for histological diagnosis, have been introduced.

Furthermore, if it were possible to differentiate UC patients with long-standing and extensive forms of colitis into subgroups with a high and a low risk of neoplasia, it would enable physicians to conduct more intensive surveillance using these modalities for patients at higher risk [2].

Protocols for the immunohistochemical analyses of Ki67 and p53 expression in fixed, paraffin-embedded tissue, are well established. Antibodies for these techniques are readily available and many laboratories are experienced in the useof such antibodies, particularly the MIB-1 antibody to Ki67 [4].

Ki67 is a huge nuclear protein that plays an important role in cellular proliferation. The monoclonal antibody Ki67 detects a nuclear antigen, which reflects cell proliferation and identifies the growth fraction of tissues and tumors [5]. Recently, Ki67 like antibodies (MIB 1) were developed that are reactive in sections of formalin fixed, paraffin wax embedded tissue after antigen retrieval.

p53 is a protein that plays an important role in regulating the cell cycle and in controlling cellular proliferation. A mutation in this protein is the most common finding in human cancers [6].

The aim of our paper was to find a more objective parameter to help distinguish regenerative changes from epithelial dysplasia and to correlate these results with the routine histological evaluation of colorectal mucosa. We were also interested in determining whether such staining techniques could be used to distinguish between low-grade dysplasia and high-grade dysplasia.

2. Material and Methods

The morphological and immunohistochemical study concerning UC related dysplasia comprised 25 cases of longstanding UC (more than 7 to10 years): 7 cases with regenerative atypia, 7 cases with low grade dysplasia, 7 cases with high grade dysplasia and 4 cases interpreted as indefinite for dysplasia as stated by the IBD Morphology Study Group criteria (1983).

The biopsies obtained during colonoscopy were fixed in formaldehyde (10%) for 24-48 hours, embedded in paraffin, then cut into 3 to 4 μ thick sections. Then, we made serial multiple sections (8-10 per case). In order to establish the pathological diagnosis and to include the case into a group of lesions, the first sections were stained using routine H&E histological methods. The examination of the H&E stained slides was made using a light microscope Carl Zeiss Ergaval type (ob. ×40, ob. ×100). The microscopic examination of these biopsies allowed us to classify and group the lesions.

From this assessment, a collection of 75 sections from biopsy specimens obtained from these patients were selected and then stained immunohistochemically to detect differences in the frequency and pattern of nuclei positive for the proliferation marker Ki67 and p53.

Three-micrometer thick sections were cut from the tissue blocks, dewaxed and rehydrated. For Ki67 immunostaining, antigen retrieval was carried out and then the sections were stained using the MIB-1 antibody to Ki67 at a dilution of 1:100, using Dako ChemMate™ reagents and detection agents as described in the manufacturer's operating manual. For p53 immunoassaying, antigen retrieval was carried out using citric acid buffer (1.05 g in 500 ml distilled water, pH 6) and microwaving at full power for 20 minutes. Sections were pretreated with 1.5% hydrogen peroxide for 15 minutes followed, after antigen retrieval, by 1:5 normal rabbit serum for 10 minutes and then the DO-7 antibody to p53 (Dako, UK) at a dilution of 1:100 for 60 minutes. Visualization was achieved by incubation with rabbit antimouse antibody (1:400) for 30 minutes. A single observer assessed all sections blindly.

Cases were divided into four groups depending on the extent of Ki67 staining: 'basal zone' (staining restricted to the basal third of the crypt); "middle zone" (extension of staining into the middle third); "top zone" (extension into the upper third); and "surface" (extension into the surface epithelium).

Two variables of p53 immunostaining were assessed: location of staining (as for Ki67), and intensity of staining - weak, moderate, strong. Weak p53 intensity staining was defined as staining which could only be detected at × 10 objective magnification and greater, strong intensity staining was defined as an equal intensity to that seen in the colorectal carcinoma used as the p53 positive control and moderate intensity staining was defined as any intensity in between.

Table 1. Extent of Ki67 IHC reaction.

Ki67 IHC staining extent	Regenerative atypia (7 cases)	Indefinite for dysplasia (4 cases)	LGD (7 cases)	HGD (7 cases)
Basal zone	2	0	0	0
Middle zone	3	2	2	0
Top zone	1	1	4	2
Surface epithelium	1	1	1	5

Table 2. Extent of p53 IHC reaction.

Extent of p53 IHC reaction	Regenerative atypia (7 cases)	Indefinite for dysplasia (4 cases)	LGD (7 cases)	HGD (7 cases)
Number of negative cases	0	1	2	1
Basal zone	2	1	1	0
Middle zone	3	1	3	0
Top zone	1	0	0	2
Surface epithelium	1	1	1	4

Table 3. Intensity of p53 staining.

Intensity of p53 staining	Regenerative atypia (7 cases)	Indefinite for dysplasia (4 cases)	LGD (7 cases)	HGD (7 cases)
Negative	0	1	2	1
Weak	4	1	3	1
Moderate	3	1	1	1
Strong	0	1	1	4

3. Results

The routine H&E staining allowed us to group the lesions into the following categories:

- **UC with regenerative atypia** (7 cases): characterized by glands surrounded by columnar epithelium with round shaped nuclei, having almost the same form and dimensions, smooth nuclear contour. Mitosis can be frequent. Unlike dysplasia, the nucleus/ cytoplasm ratio from the regenerative cells is low and the cell cytoplasm is intensely eosinophilic. The presence of cryptic abscesses or cryptitis pleads in favor of the reactive alteration diagnosis.
- **UC indefinite for dysplasia** (4 cases): this diagnosis was used for more severe cellular alterations of the lesions than those in active colitis; however they were not relevant to a final diagnosis of dysplasia.
- **Low grade dysplasia UC** (7 cases): characterized by mucosal alterations which resemble those of adenomas. The glandular crypts are surrounded by tall, oval shaped, pseudostratified epithelial cells with hyperchromatinic nuclei. Most of these cells are at the base of the crypt. In the luminal surface, these cells do not differentiate themselves into goblet cells or absorptive cells. The mucous secretion, although present, is quantitatively reduced. Dystrophic mucous cells might be present, too. Relatively frequent mitoses are met all along the full length of the crypt. The somehow basal polarity of the nuclei is an important diagnostic criterion for low grade dysplasia. Low grade dysplasia might be associated with endocrine cells hyperplasia and with the Paneth cell metaplasia. The glandular architecture is normal or slightly modified.
- **High grade dysplasia UC** (7 cases): This is diagnosed based on complex architectural alterations consisting of crowded glands, irregularly shaped, branched, and situated back to back. Cellular atypia are more obvious than in low grade dysplasia. Tall or cubic cells present a discrete nuclear stratification with oval or round shaped nuclei, which are both hyperchromatinic and pleomorphus, with prominent nucleoli. The epithelial cells resemble the invasive carcinoma cells and are less alike the tall, regulated shaped adenoma cells. Unlike low grade dysplasia where the unvaryingly, regular shaped nuclei are situated in the basal half of the cell, in high grade dysplasia, the nuclei go beyond the middle part of the cell reaching the apical edge.

Figure 1. Ki67 intense nuclear staining in the inferior basal 1/3 of the glandular crypts (DAB).

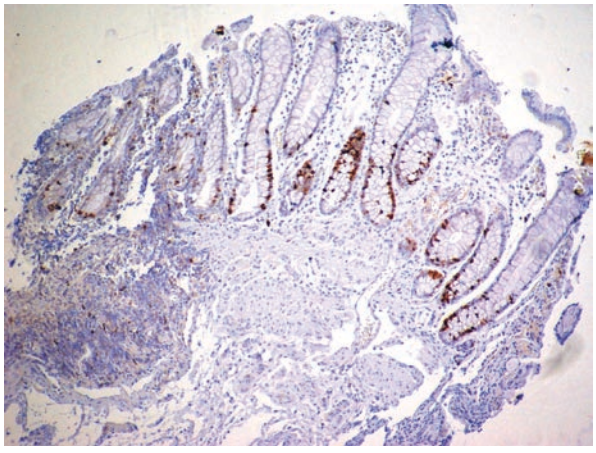


Figure 2. Ki67 intense staining in the germinative centers of the lymphoid follicles and basal glands (DAB).

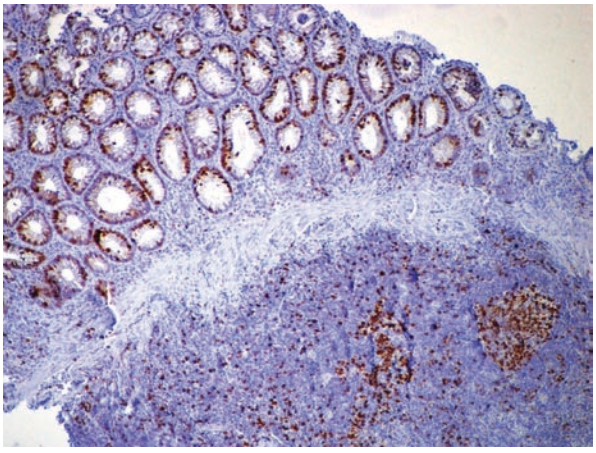


Figure 3. The extension of the proliferative compartment towards the inferior third of the colonic crypts - Ki67 (DAB).

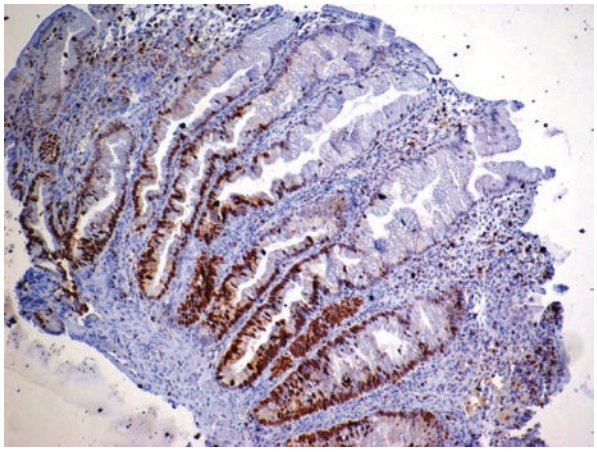
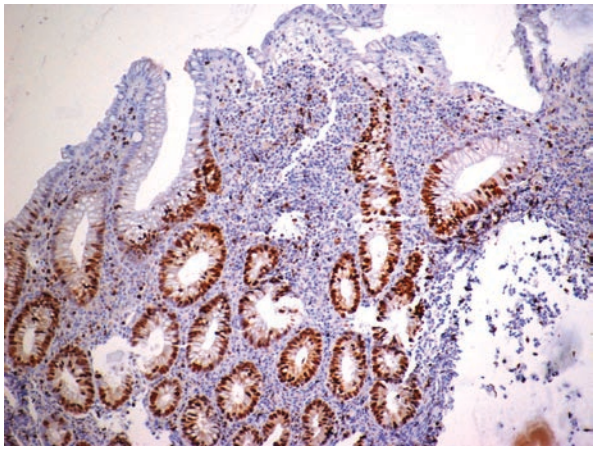


Figure 4. Ki67 immunostaining into the basal and middle third of the colonic crypts (DAB).



In order to improve the positive diagnosis of UC related dysplasia and to differentiate regenerative atypia from dysplasia as well as low grade dysplasia from high grade dysplasia, we applied Ki67 and p53 antibody immunoreactions to every studied case.

The immunohistochemical assaying with Ki67 antibody:

The cells were considered Ki67 positive in the presence of nuclear granular or diffuse brown staining. Mitosis cells presented both nuclear and cytoplasm staining. The Ki67 hall-mark indices were used to quantify the reaction in the form of the number of Ki67 positive cells reported 500 Ki67 positive and, respectively negative tumor cells.

All the colorectal mucosal fragments showed an intense and relatively homogenous nuclear staining in the inferior 1/3 of the crypts and into the germinative centers of the lymphoid follicles seen in a section (Figure 1 and 2).

In our study, we observed the site of the stained nuclei along the colonic crypts and at the surface epithelium. According to the pattern of Ki67 staining, our cases were classified into the following:

- "Basal zone" (the presence of the stained nuclei restricted to the basal third of the crypt)
- "Middle zone" (stained nuclei extension into the inferior and middle third of the colonic crypts- Figure 3 and 4)
- "Top zone" (the existence of positive nuclei in the upper third of the crypts- Figure 5 and 6)
- "Surface" (extension of the staining into the surface epithelium- Figure 7 and 8)

The immunohistochemical staining patterns were diverse in the colonic crypts that we studied.

All 4 types of Ki67 staining, including surface "staining," were seen within the regenerative atypia cases. The regenerative atypia cases showing "surface" Ki67 staining were characterized by particularly marked

Figure 5. Ki67 immunostaining into the superficial part of an atrophic glandular crypt. (DAB).

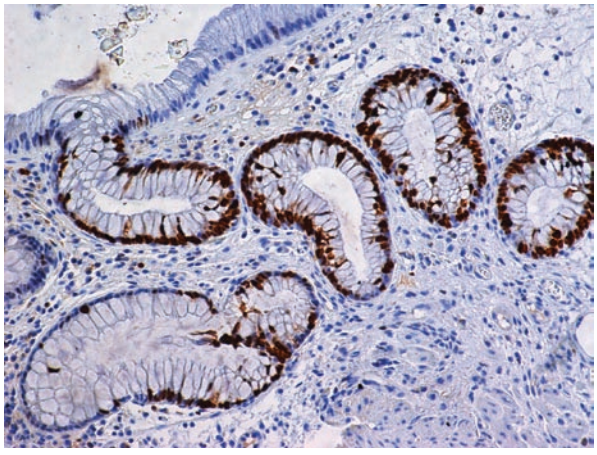


Figure 6. Ki67 positive immunostaining in the superficial part of the crypt (DAB).

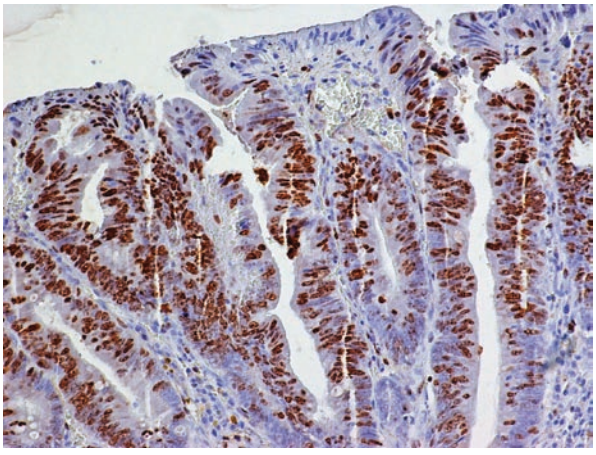


Figure 7. Extension of the Ki67 staining into the surface epithelium (DAB).

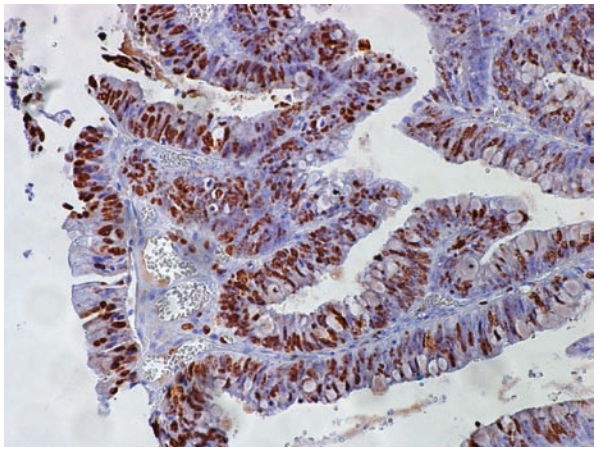
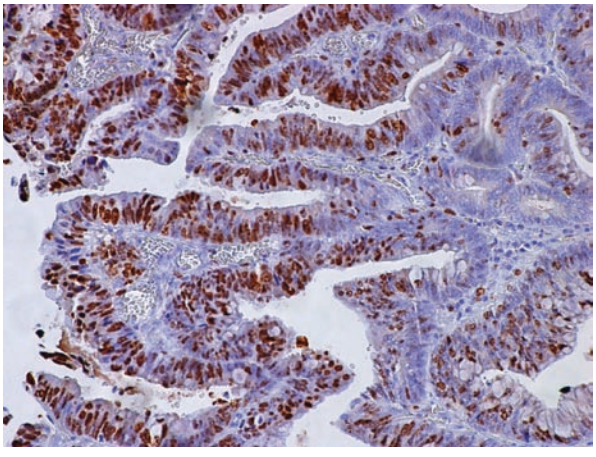


Figure 8. "Surface" staining of Ki67 (DAB).



acute inflammation with focal surface epithelial erosion. In conclusion,

- there were important differences between the percentages of positive Ki67 cells in severe dysplasia comparative to the regenerative epithelium.
- in low-grade dysplasia, the immunoreactivity of Ki67 was more pronounced in the extended basal zone being only rarely observed at the epithelial surface; some of the cases showed staining restricted to the lower two thirds of the crypt, but none of the high-grade dysplasia cases showed this aspect (Figure 9).
- in high-grade dysplasia, Ki67 was diffuse throughout the crypts.
- none of the dysplasia cases, compared with regenerative cases, showed Ki67 staining restricted to the basal third of the crypt (Figure 10).

3.1. The immunohistochemical assaying with p53 antibody

The over expression of p53 oncoprotein might show one of the most frequent genetic mutations described in the colorectal carcinogenesis. In our study, we used monoclonal antibody DO7, which detects both the savage and mutant forms of p53 protein.

In sporadic colorectal cancer and UC-associated cancer, p53 protein over expression is a characteristic finding (Figure 11 and 12). None of the regenerative atypia cases showed strong intensity p53 staining compared to dysplasia cases (Figure 13). From regenerative epithelium cases showing p53 staining, only 1 case showed extension of staining into the upper third of the crypt (moderate intensity) and only 1 case showed extension onto the surface (weak intensity). None of the high grade dysplasia cases showed restricted staining to the lower two thirds of the crypt.

Figure 9. Low-grade dysplasia – Ki67 (DAB).

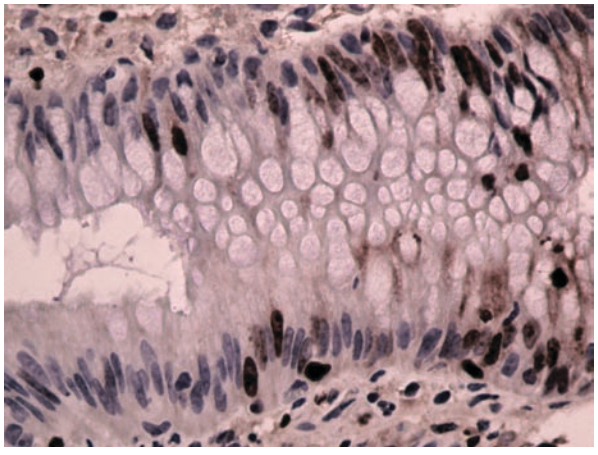


Figure 10. Dysplastic UC (DAB).

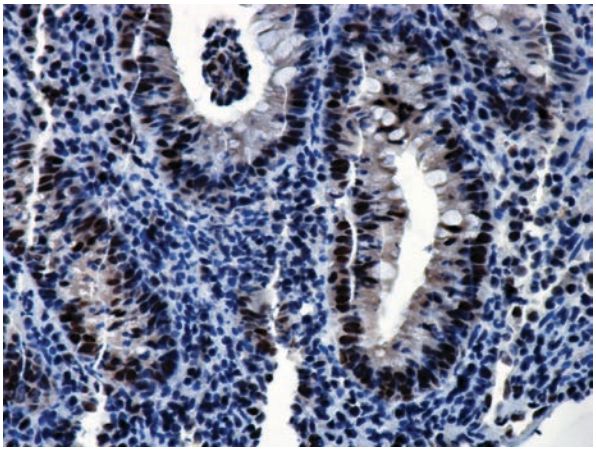


Figure 11. Sporadic colorectal cancer - p53 (DAB).

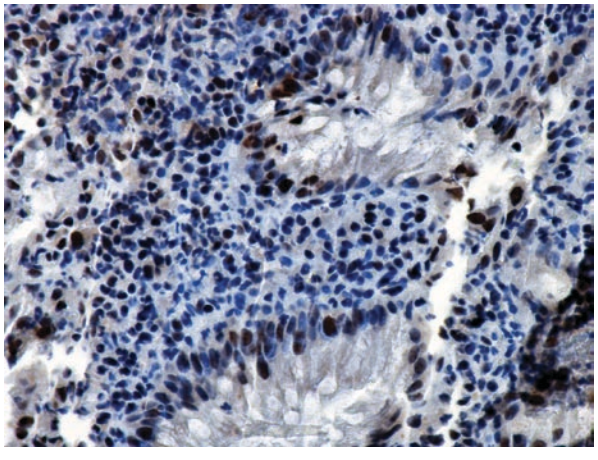


Figure 12. UC-associated cancer - p53 (DAB).

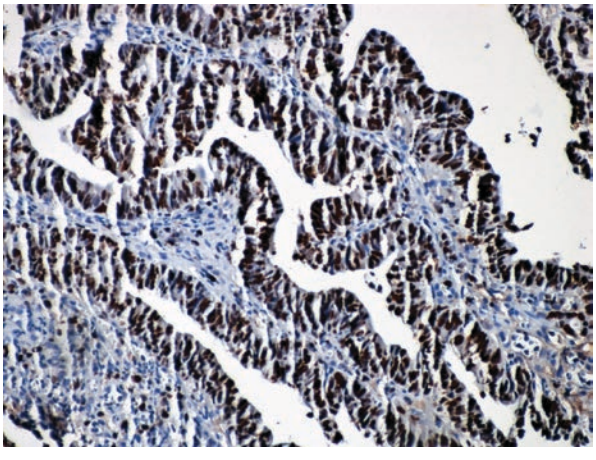
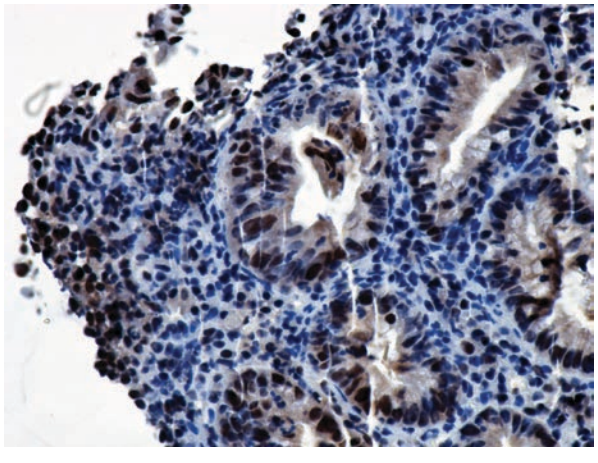


Figure 13. Regenerative atypia cases - p53 (DAB).



All the cases of high-grade dysplasia showed extension of Ki67 and p53 staining above the basal two thirds of the crypt.

In general, the histological remodeling changes correlated well with the changes encountered while studying the 2 immunohistochemical markers.

4. Discussion

We investigated whether Ki67 and p53 immunostaining can be used to aid the diagnosis and grading of UC-related dysplasia.

The increased risk of development CRC in ulcerative colitis is well reported and a frequently encountered dilemma is the diagnosis and assessment of genuine dysplasia in cases of longstanding ulcerative colitis. This increase incidence of cancer seen in UC patients might be related to an increased cellular proliferation within the mucosal crypts because of recurrent or persistent inflammation; the risk of CRC increases with the duration of the UC. In time, inflammation may give rise to epithelial changes known as dysplasia. Epithelial

regeneration in inflammation can be irregular or bizarre and imitate dysplasia; or even worse, disguise the neoplastic or preneoplastic changes [7].

It is difficult to separate benign regenerative epithelial atypia true dysplasia, using histological criteria alone. In addition, it is difficult then to assess the grade of dysplasia once confirmed. Ki67 and p53 expression may offer an objective means of doing this.

Only a few reports have evaluated immunohistochemically Ki67 protein in UC-associated dysplasia with the purpose of determining the characteristics of cell proliferation, and its malignant potential [8-11].

The main finding of our study was the highly significant differences in percentages of cells with Ki67 immunostaining in high grade and in low-grade dysplastic lesions, compared with normal epithelium and epithelium with regenerative changes. We found 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 towards the luminal surface, where they are sloughed off. These aspects were also found in previous studies [12]. Increased cell proliferation is a well recognized finding in areas of healing and active colitis. Indeed, the regenerative atypia cases which showed 'diffuse' staining showed particularly marked acute inflammation [4].

Deschner [12] and Lipkin [13] were the first to find abnormal, proliferative cellular lesions in colonic crypts and in animal models and in humans with familial adenomatous polyposis, sporadic adenomas and carcinomas, as well as in ulcerative colitis [11]. They concluded that increased DNA synthesis might be an important step in a final common pathway leading to malignant transformation.

Alpers et al found that patients with long standing ulcerative colitis of more than 10 years' duration showed a lack of inhibition by phosphodiesterase inhibitors on thymidine incorporation into DNA in cultured colonic tissue [14]. They concluded that the duration of inflammation, rather than the extent of the disease within the colon, might be the more important factor leading to alteration in the control of DNA synthesis [1]. Considering their conclusion as a starting point, we enrolled only longstanding ulcerative colitis cases into our study, hoping to demonstrate this same aspects.

Noffsinger et al, [15] elucidated that in some dysplasias, and all carcinomas, Ki67 is diffusely distributed throughout the crypts, suggesting complete deregulation of normal cell proliferation. While Noffsinger and colleagues stated that some of their nondysplastic cases showed 'foci of active inflammation' and found focal surface epithelial staining in three of their nondysplastic cases, no comment was made as to whether the 2 were related. We have confirmed the findings of these previous studies that restriction of staining to the basal third of the crypt is not seen in cases of UC-related dysplasia.

Two other studies used the MIB-1 antibody to detect the percentage Ki67 stained nuclei in relation to the distribution throughout the mucosal crypts in UC-associated dysplasia [16,17]. In our study, Ki67 was found following the same pattern and showing diffuse distribution in cases of high grade dysplasia.

Most p53 studies in ulcerative colitis have shown that the frequency of immunohistochemical overexpression and point mutation increases with the severity of dysplasia and this suggests that it is probably a late event [18-21]. One important function of p53 lies in its role in the induction of apoptosis. The reduction in the ability to undergo apoptosis may lead to immortalization of cells. This would tend to increase the accrual of mutations, which may eventually be sufficient for malignant transformation [5].

In sporadic colorectal cancer and UC-associated cancer, p53 protein overexpression is a characteristic finding, and there is a high concordance with p53 mutations [22]. In conclusion, we emphasize that p53 immunostaining is a good marker for assessing genetic alterations that precede histologically malignant change and for diagnosing carcinoma in UC or other colorectal diseases. In the same time, given the high degree of sensitivity of antigen retrieval methods, in our study we might have detected overexpression of wild type protein - that is, appropriate expression of non-mutant p53 - in an attempt to induce apoptosis in neoplastic cells.

We have demonstrated that restriction of Ki67 or p53 staining to the lower two-thirds of the crypt excludes a diagnosis of high grade dysplasia.

The negative predictive value of p53 staining, for excluding a diagnosis of high grade dysplasia, is twice that of Ki67 staining but not much lower than that of the two tests combined [4].

Traditional histopathological evaluation of dysplasia in long standing ulcerative colitis is often difficult, especially when deciding whether a lesion shows low grade dysplasia, regenerative changes, or is indefinite for dysplasia [23]. We recommend that assessment of both the percentage of positive cells, and the staining pattern, should be made. We also stress that objective findings, such as p53-protein over expression, the Ki67, and morphometric values should be used to evaluate the cytological progression and invasiveness of both UC-associated carcinoma and common type colorectal cancer.

A new study with a larger patient population might show whether Ki67 and p53 based classification of mucosal alterations related to longstanding ulcerative colitis is a more objective way of predicting malignant transformation than traditional histopathological evaluation alone.

5. Conclusions

- Restriction of Ki67 staining to the basal third of the crypt appears to exclude a diagnosis of dysplasia, whereas strong intensity p53 staining suggests a diagnosis of dysplasia.
- Ki67 and p53 restriction in the two thirds of the crypt appears to exclude the diagnosis of high grade dysplasia.
- Assessment of the number of Ki67 and p53 immunostained cells is of additional value in deciding whether the mucosa is regenerative or dysplastic. Thus, this technique could be combined with routine histological evaluation of colorectal mucosa in order to improve the diagnostic accuracy and to appreciate the risk of malignant transformation.

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