

## Ki-67: a useful marker for the evaluation of dysplasia in ulcerative colitis

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### Abstract

**Aims**—Evaluation of dysplasia in long standing ulcerative colitis is a difficult and often subjective task. Therefore, the aim of this study was to search for a more objective parameter to help distinguish regenerative changes from epithelial dysplasia.

**Methods**—A total of 97 sections from colectomy specimens from 12 patients with ulcerative colitis of more than 10 years duration were stained immunohistochemically with MIB 1 to detect differences in the frequency and pattern of nuclei positive for the proliferation marker Ki-67. All patients had epithelial dysplasia in one or more areas (high grade dysplasia, n = 16; low grade dysplasia, n = 15; indefinite for dysplasia, n = 16), and three patients had additional adenocarcinoma (one Duke's C multifocal, mucinous carcinoma; one Duke's C adenocarcinoma in the sigmoid; and one Duke's A adenocarcinoma in the caecum). Two patients had adenomas—one had an 8 cm villous adenoma with intramucosal carcinoma, and the other had a 4 cm tubulovillous adenoma with high grade dysplasia.

**Results**—There were highly significant differences between the percentages of Ki-67 immunopositive cells in low grade and high grade dysplasia and carcinoma compared with regenerative epithelium. In high grade dysplasia and carcinoma, the distribution of Ki-67 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.

**Conclusions**—Assessment of the number of Ki-67 immunostained cells is of additional value in deciding whether the mucosa is regenerative or dysplastic, and the MIB 1 staining pattern is characteristic for most lesions with high grade dysplasia and carcinoma. Therefore, this technique could be combined with routine histological evaluation of colorectal epithelium being examined for dysplasia.

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**Keywords:** Ki-67 immunohistochemistry; ulcerative colitis; dysplasia

Extensive and long standing ulcerative colitis of more than eight years duration is well known to predispose patients to the development of colorectal carcinoma.<sup>1-4</sup> This increase

in the incidence of cancer might be related to increased cellular proliferation within the mucosal crypts as a result of recurrent or persistent inflammation, and the risk of cancer increases with the duration of the disease.<sup>5</sup> In time, inflammation may give rise to epithelial changes known as dysplasia. Despite attempts to standardise the criteria for the classification of dysplasia,<sup>6</sup> evaluation and grading of dysplasia in the colorectum is a difficult and partly subjective task. Epithelial regeneration in inflammation can be irregular or bizarre and imitate dysplasia; or even worse, disguise the neoplastic or preneoplastic changes.

In previous studies, the use of tritiated thymidine and the non-radioactive thymidine analogue, bromodeoxyuridine, has demonstrated increased labelling indices in apparently normal rectal mucosa in patients at high risk of developing colorectal carcinoma.<sup>7,8</sup> The monoclonal antibody Ki-67 detects a nuclear antigen that reflects cell proliferation, thus identifying the growth fraction of tissues and tumours.<sup>9</sup> Recently, Ki-67 like antibodies (MIB 1-3) were developed that are reactive in sections of formalin fixed, paraffin wax embedded tissue after antigen retrieval.<sup>10-12</sup>

Shepherd and colleagues<sup>13</sup> evaluated the growth fraction in colorectal carcinomas using frozen sections and this was followed by several studies of carcinomas and adenomas.<sup>14-17</sup> Until recently, there have been few studies of Ki-67 antigen in relation to regeneration and dysplasia in ulcerative colitis.<sup>18,19</sup> The aim of our study was to try to validate these results.

Our material comprised 30 colectomy specimens collected from patients with ulcerative colitis of long duration—most of them having epithelial dysplasia, and some with carcinoma. This gave us an opportunity to study Ki-67 reactivity in epithelial changes from regeneration through low and high grade dysplasia to carcinoma, and offered important information about proliferation related changes that might be of prognostic importance during successive stages of epithelial transformation.

### Methods

#### PATIENTS AND COLECTOMY SPECIMENS

From our total of 30 colectomy specimens, 12 patients who had undergone colectomy between June 1992 and September 1994 were chosen. These patients suffered from long standing (10-30 years) ulcerative colitis with different types of epithelial changes, including dysplasia and cancer. All specimens showed more or less inactive colitis with little active inflammation. Of these 12 patients, 10 were men and two were women, (median age, 41 years; range, 29-75). Three had

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Table 1 Clinical and pathological data of patients with ulcerative colitis

Patient	Age	Sex	Duration (years)	Carcinoma Dukes's stage/other tumour	Localisation of tumour	Max. grade of dysplasia
1	75	M1	16	C multifocal	T/L colon	High grade
2	41	M	25	C	Sigmoid	Low grade
3	31	M	16	A	Caecum	High grade
4	40	M	26	V tumour intramuc. carc.	T colon	Low grade
5	64	F	13	Tub. high grade	Rectum	High grade
6	41	M	11			High grade
7	39	M	16			High grade
8	41	M	24			High grade
9	46	M	30			High grade
10	38	M	20			Low grade
11	45	M	10			Low grade
12	29	F	15			Low grade

A, Dukes's stage A adenocarcinoma; C, Dukes's stage C adenocarcinoma; T/L colon, transverse and descendant colon; T colon, transverse colon; Tub, tubulovillous adenoma; V tumour intramuc. carc., villous adenoma with intramucosal carcinoma.

adenocarcinomas—one had a Dukes's A carcinoma in the caecum, another had Dukes's C carcinoma in the sigmoid, and the third patient presented with a multifocal and mucinous Dukes's C carcinoma with three tumours in the transverse and descendant colon. One patient had a large, villous adenoma (diameter, 8 cm), with intramucosal carcinoma in the transverse colon, and one patient had a tubulovillous adenoma (4 cm) with high grade dysplasia in the rectum (table 1).

#### IMMUNOHISTOCHEMICAL ANALYSIS

On average, 25 sections were taken throughout the large bowel wall of each colectomy specimen. These were fixed in buffered formalin and stained with a trichrome routine method containing haematoxylin, azofloxine, and saffron.<sup>20</sup> Based on light microscopy, seven to 10 sections were chosen to cover the spectrum of epithelial alterations in each patient, a total of 97 sections altogether. No area was found to be completely normal in any of the colectomy specimens. Therefore, normal control mucosa was scored in eight biopsies from patients who had undergone colonoscopy because of uncharacteristic bowel problems, but who did not have mucosal changes. There were 43 sections with regenerative epithelium. The other groups were: "epithelium indefinite for dysplasia, probably positive" (n = 16); low grade dysplasia (n = 15); and high grade dysplasia (n = 16). From five carcinomas in three patients, seven sections were taken (table 2). There was little active inflammation, and all sections, except those from carcinomas and adenomas, showed atrophy of the mucosa, with branched crypts and some chronic inflammation.

Serial sections cut at 5 µm thickness were obtained for immunohistochemical evaluation and placed on pretreated, gelatine coated slides. After deparaffinising with xylene, the sections were rehydrated through graded alco-

Table 2 Epithelial changes and Ki-67 immunoreactivity in patients with ulcerative colitis

Histological classification	No. of sections	Basal	Expanded basal zone	Extension to surface	Diffuse
Regenerative	43	0	43	0	0
Indefinite for dysplasia	16	0	14	2	0
Low grade dysplasia	15	0	11	3	1
High grade dysplasia	16	0	3	2	11
Carcinoma	7	0	0	0	7
Total	97				

hols to water. The slides were then incubated in 10 mM citrate buffer (pH 6.3) in a microwave oven (750 W) for three periods of five minutes. After incubation, the sections were cooled for 30 minutes at room temperature, and then washed in Tris buffer for five minutes. The sections were then incubated overnight (18 hours) at room temperature with the mouse monoclonal antibody MIB 1 (a gift from Dr J Gerdes, Germany). This antibody recognises Ki-67 epitopes in formalin fixed material. Both positive and negative (omitting primary antibody) control reactions for each series of immunostained sections were carried out. Staining was performed with the conventional alkaline phosphatase anti-alkaline phosphatase method, using fast red (Sigma Chemical Co, St Louis, Missouri, USA) as chromogen. The slides were lightly counterstained with haematoxylin.

Cells were scored as positive when nuclear staining was evident. Slides were chosen where crypts were well orientated, and the total number of cells in whole crypts was always counted up to the mucosal surface. The number of crypts included depended on the grade of atrophy in that definite region. A pilot experiment showed that counting of about 500 cells gave a valid percentage of positive cells that did not change significantly from that of scoring up to 1000 cells. The percentage of positive cells was registered for each region and tabulated. In addition, we classified each lesion according to the pattern of Ki-67 immunoreactivity. We distinguished between four patterns: basal, when the proliferating cells were confined to the basal one third of the crypt; expanded basal zone, when Ki-67 positive cells extended beyond the basal one third of the crypt; extension to surface, when positive cells presented on the mucosal surface; and diffuse, when proliferating cells were present at all levels within the crypt, as well as on the mucosal surface. These are similar to the categories proposed by Noffsinger *et al.*<sup>19</sup>

#### STATISTICAL METHODS

To account for possible correlations between observations from the same patient, we used a variance component model with "patient" as random component and "epithelial grading" as "fixed effect". We used the "proc mixed" procedure of the SAS statistical software package.<sup>21</sup> The p values were Bonferroni corrected. The Kappa test<sup>22</sup> was used to test for intraobserver and interobserver reproducibility. A p value < 0.05 was considered to be significant.

## Results

#### EVALUATION OF EPITHELIAL CHANGES

Two independent and experienced histopathologists (SNA and OPFC) evaluated independently 59 of the 97 routinely stained slides for different grades of dysplasia according to standard criteria for comparison.<sup>6</sup> The remainder of the slides were classified by SNA alone and afterwards agreed upon by OPFC. Interobserver agreement was substantial (Kappa = 0.64), and intraobserver agreement

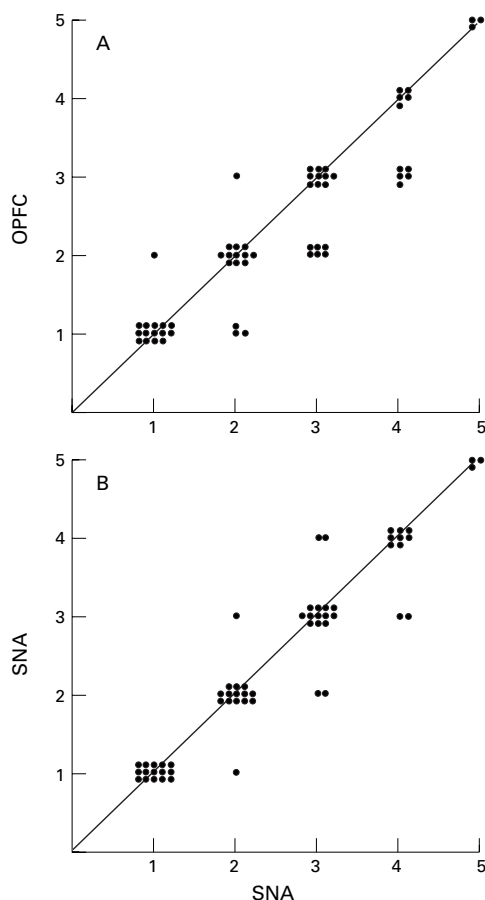


Figure 1 Scatter diagrams of interobserver and intraobserver reproducibility. The degree of accordance for grading of epithelial changes (A) between the first observer SNA and the second observer OPFC ( $P_o = 0.73$ ;  $Kappa = 0.64$ ), and (B) between two observations by the same histopathologist (SNA), the last performed blindly after 12 weeks ( $P_o = 0.86$ ;  $Kappa = 0.82$ ).

after three months was “almost perfect” ( $Kappa = 0.82$ ) (fig 1).

COUNTING OF KI-67 POSITIVE CELLS

Of the 97 sections, 43 showed regenerative changes with little active inflammation, 16 were indefinite for dysplasia, 15 showed low grade

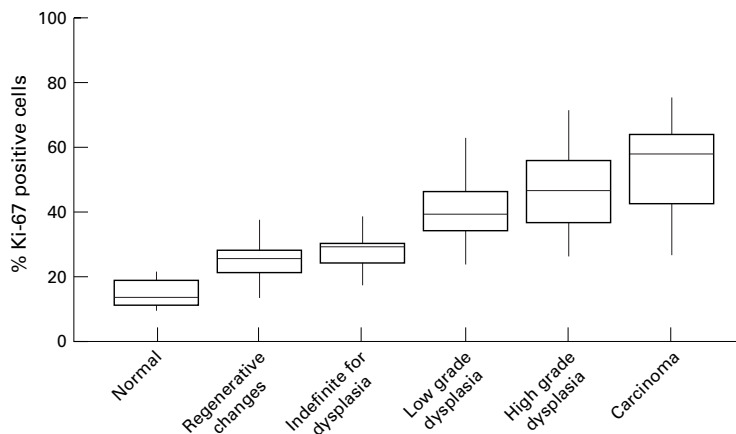


Figure 2 Box plot illustrating percentage of Ki-67 positive cells in different epithelial lesions in ulcerative colitis: normal epithelium, regenerative epithelium with little active inflammation, epithelium indefinite for dysplasia, low grade dysplasia, high grade dysplasia, and carcinoma. The horizontal lines in the boxes indicate the mean.

dysplasia, 16 showed high grade dysplasia, and seven slides were from the five carcinomas examined.

The sections showed varying numbers of cells with positive nuclear staining (table 2) and negligible background staining. Most cells stained strongly positive, and less than 10% on average were stained less intensely. All cells with staining intensity above background were scored as positive for Ki-67. The statistical analysis showed that the groups differed significantly ( $p$  values  $< 0.001$ ). We calculated the Bonferroni corrected comparison between groups. The numbers of Ki-67 positive cells were significantly higher in areas with low grade dysplasia (mean (SD), 41.6 (12)), high grade dysplasia (mean (SD), 47.4 (13)), and carcinoma (mean (SD) 53.9 (17)) compared with regenerative epithelium (mean (SD), 25.7 (6)), (all  $p$  values  $< 0.001$ ; fig 2), as well as low grade dysplasia, high grade dysplasia, and carcinoma compared with normal epithelium (mean (SD) 14.8 (4.3);  $p < 0.001$ ). Epithelium with low grade dysplasia had significantly higher numbers of immunopositive cells compared with both epithelium indefinite for dysplasia and regenerative epithelium ( $p < 0.001$ ), whereas the number of Ki-67 positive cells in epithelium indefinite for dysplasia was not statistically different from that in regenerative epithelium ( $p \approx 1$ ). Epithelium indefinite for dysplasia had significantly more positively stained nuclei compared with normal epithelium ( $p = 0.009$ ). Comparing high grade dysplasia with low grade gave a  $p$  value of 0.19, and positive staining in high grade dysplasia was almost equal to that in carcinoma. Carcinoma differed from low grade dysplasia ( $p = 0.03$ ), as did regenerative compared with normal epithelium ( $p = 0.03$ ). Examples of positive staining with MIB 1 in regenerative epithelium, in low and high grade dysplasia, and in carcinoma are shown in fig 3.

KI-67 STAINING PATTERNS

Table 2 shows patterns of Ki-67 immunoreactivity in non-neoplastic and neoplastic lesions. All sections with regenerative changes had expansion of the basal zone (43 of 43) compared with that which is normally seen in colonic mucosa. Most high grade dysplasias (11 of 16) and all sections from carcinomas (7 of 7) showed diffuse and scattered staining along the full length of the crypts. The remainder of the high grade dysplasias showed either extension of staining to the surface (2 of 16) or an expanded basal zone (3 of 16). In both low grade dysplasia and epithelium indefinite for dysplasia, most lesions had an expanded basal zone (11 of 15 and 14 of 16, respectively). Three lesions with low grade dysplasia had extension of Ki-67 positive cells up to and in to the surface epithelium, and one single lesion showed diffuse staining along the crypts.

Discussion

The main finding of our study was the highly significant differences in percentages of cells with Ki-67 immunostaining in high grade and low grade dysplastic lesions and carcinoma,



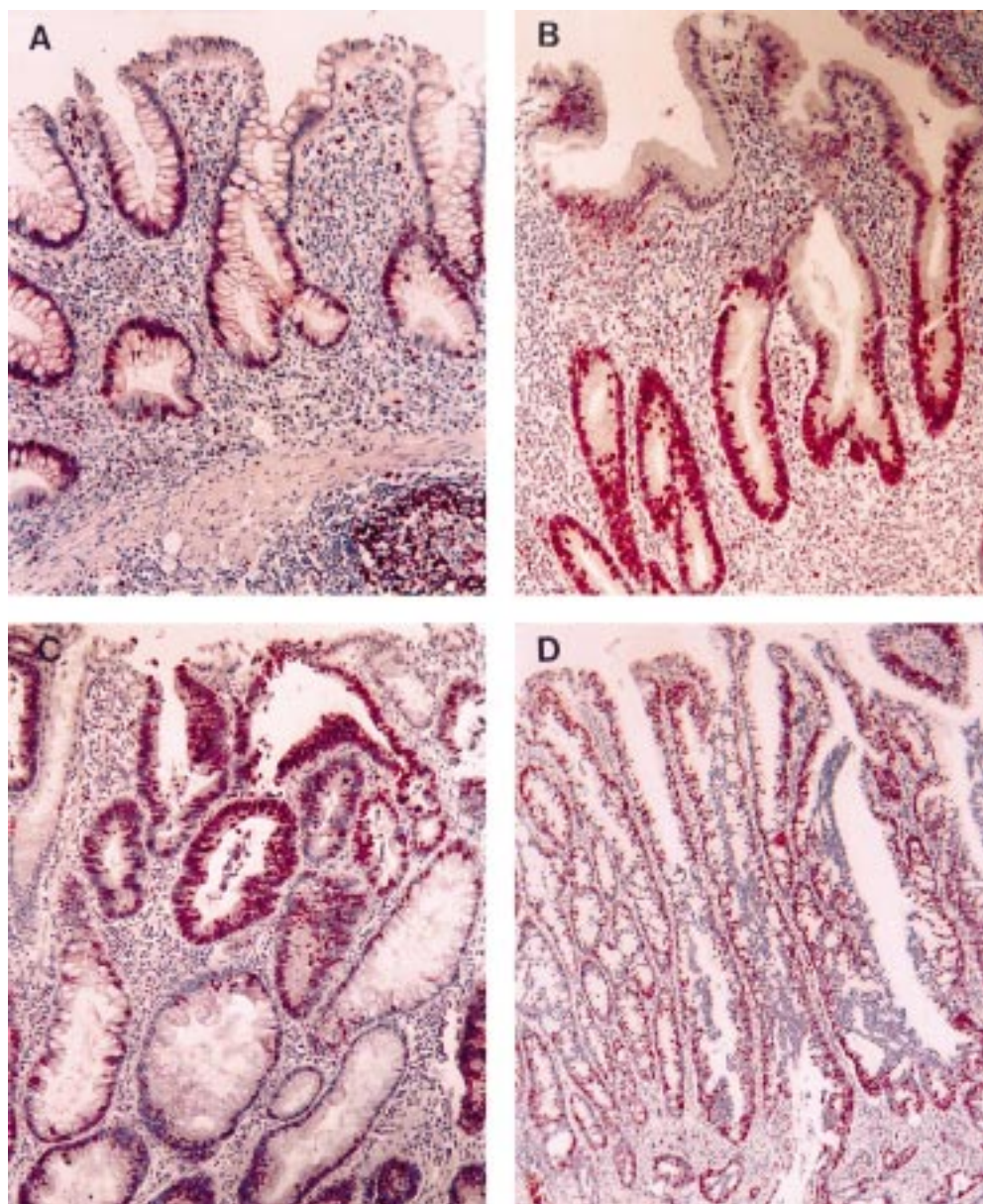


Figure 3 Different patterns of Ki-67 immunoreactivity. (A) Expanded basal zone in a section with regenerative epithelium, with positive cells localised mainly to the bottom and middle section of the crypt. (B) Low grade dysplasia shows positive cells throughout the full length of the crypts, with expansion almost to the top, whereas in high grade dysplasia (C), positive cells are marked at the top of the mucosa and are scattered throughout the lesion. (D) In carcinoma, the cells are stained diffusely in all compartments of the crypts, as well as in the surface epithelium and the infiltrating crypts.

compared with normal epithelium and epithelium with regenerative changes ( $p < 0.001$  for each of the groups). Regenerative epithelium was not different from epithelium indefinite for dysplasia ( $p \approx 1$ ) with respect to Ki-67 positivity.

In normal colonic mucosa, the predominant area of cell proliferation is localised 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.<sup>23</sup> Deschner<sup>23</sup> and Lipkin<sup>24</sup> were the first to find abnormal, proliferative cellular lesions in colonic crypts in animal models and in humans with familial adenomatous polyposis, sporadic adenomas, and carcinomas, as well as in ulcerative colitis.<sup>25</sup> They concluded that increased DNA synthesis might be an important

step in a final common pathway leading to malignant transformation.

Biasco *et al* used tritiated thymidine in their cell kinetic studies of non-neoplastic rectal epithelium in patients with ulcerative colitis, and compared the results with those from patients with multiple sporadic adenomas and control subjects without colonic disease.<sup>26</sup> The labeling index was similar in the three groups, but there was an overall increase in the pool of proliferating cells in patients with ulcerative colitis, especially in the upper 40% of the crypts. 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.<sup>27</sup> 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.

Several authors have found that the proliferative compartment in adenomas is expanded or misplaced, often reaching to the mucosal surface.<sup>14-28</sup> The proliferating cells are distributed uniformly throughout the section, with accentuation at the top.

Because there is an almost linear increase in the Ki-67 reactivity from normal colorectal mucosa, through regeneration and various grades of dysplasia, to carcinoma (fig 2), our results clearly confirm the importance of increased cell proliferation for the progression to malignancy in this context. Furthermore, this process is also associated with a disruption of the normal structural organisation of the growth compartment, because most high grade dysplasias and all cancers had a diffuse staining pattern with scattered Ki-67 positive cells (table 2; fig 3).

Several studies have shown that there is a very close association between Ki-67 immunoreactivity and the cell cycle. Expression appears in mid to late G1, rising through S phase and G2 to reach a maximum during mitosis.<sup>29</sup> After mitosis, the antigen is degraded rapidly, with a half life of an hour or less for the detectable antigen.<sup>30</sup> There is strong evidence that Ki-67 expression correlates with cell proliferation, although it consistently overestimates the growth fraction, as measured by S and G2 fractions.<sup>31</sup>

In the development of sporadic colorectal carcinomas, mutations in the tumour suppressor gene APC and in the protooncogene Ki-ras are relatively early events that are assumed to be related to increased cell proliferation.<sup>32</sup> However, because there are conflicting reports on the frequency of mutations within these genes during tumour development in ulcerative colitis,<sup>33-35</sup> such mutations are not likely to be able to explain the increased proliferation in dysplastic development seen in ulcerative colitis. Putative candidate genes involved in this process would be those encoding cyclins, cyclin dependent kinases, and their inhibitors.

Two recent studies also used the MIB 1 antibody in formalin fixed, paraffin wax embedded colonic tissue to detect Ki-67 positive nuclei in dysplastic and non-dysplastic lesions from patients with inflammatory bowel disease.<sup>18-19</sup> Kullmann *et al* found labelling indices significantly higher in high grade dysplasia compared with low grade dysplasia in both upper and lower compartments, and significantly higher in the superficial part in sections "indefinite for dysplasia, probably positive", compared with normal colorectal tissue.<sup>18</sup> This agrees with our results. However, they could not show significant differences between the group that were "indefinite for dysplasia, probably positive" and low grade dysplasia, assuming that this could be caused by active inflammation. In our sections from patients with long standing ulcerative colitis very few patients had active inflammation. In our study, cells were counted along whole

crypts up to the mucosal surface, without dividing them into compartments. In long standing colitis, the mucosa is often atrophic, with branching and distorted crypts not reaching the muscularis mucosa. In our opinion, this makes division of the crypts difficult to perform in a reproducible way. The fact that we had similar Ki-67 indices in regenerative epithelium and epithelium indefinite for dysplasia, whereas the latter category had Ki-67 indices similar to low grade dysplasia when scored by Kullmann and colleagues,<sup>18</sup> might be a result of consistent differences in the sub-classification of dysplasias.

Noffsinger *et al* concluded that dysplastic lesions and carcinomas show a different Ki-67 staining pattern 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.<sup>19</sup> We also investigated the staining pattern in our material and found similar distributions in high grade dysplasias and carcinomas, as did these authors, but almost all lesions in our material showed varying degrees of expanded basal zones, with the exception of the carcinomas. Therefore, we recommend that the total percentage of Ki-67 stained cells should also be taken into account.

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. To assure the reproducibility of this method, we recommend that assessment of both the percentage of positive cells, and the staining pattern, should be made. Our study is too small to predict the outcome for any definite patient. A new study with a larger patient population might show whether Ki-67 based classification of mucosal alterations related to long standing ulcerative colitis is a more objective way of predicting malignant transformation than traditional histopathological evaluation alone.

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