Computer-assisted quantitative analysis of Ki-67 antigen in dysplasia – associated lesions or masses in ulcerative colitis

Komjuterska kvantitativna analiza Ki-67 antigena u lezijama udruženim sa displazijom ili masama kod ulceroznog kolitisa

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Background/Aim. The aim of this study was to apply computer-assisted methodology in assessment of Ki-67 positivity in “adenoma-like” dysplasia associated lesions or masses (DALMs), and carcinoma in ulcerative colitis (UC), and to determine a new approach to grading of Ki-67 staining intensity. Methods. Immunohistochemical slides were quantitatively analyzed for estimation of proportion and intensity of Ki-67 positive-stained cells in a total of 50 “adenoma-like” DALMs (27 with low-grade dysplasia and 23 with high-grade dysplasia), and 17 adenocarcinomas associated with UC. The four grades of immunohistochemical staining intensity were established by an automated classification of nuclear optical densities. Results. The Ki-67 labeling index (LI) in low-grade dysplasia was significantly lower than in high-grade dysplasia (p < 0.001). The Ki-67 LI of carcinomas was not significantly different from the value obtained in high-grade dysplasia, however having the difference in percentage values of the moderate stained nuclei (p < 0.05). The overall average values of chromogene nuclear optical densities, showed statistically significant differences between DALMs and carcinoma (p < 0.05), although not between normal mucosa and low-grade dysplasia (p > 0.05). Conclusions. The obtained results imply, according to the overall percentage of labeled nuclei, that high-grade dysplasia is very close to carcinoma, while there is the difference in the percentage of moderately stained nuclei. We showed that Ki-67 positivity have a different internal distribution which could be useful in analysing these lesions. These findings also, indicate the important biological differences between low-grade dysplasia and carcinoma in UC, and a low proliferative potential of the former. Automated image analysis permits an objective estimation of Ki-67 immunohistochemical staining in UC-associated dysplasia and carcinoma.

Key words: colitis, ulcerative; polyps; carcinoma; computers; immuno histochemistry; Ki-67 antigen.

Apstrakt

Uvod/Cilj. Cilj ovog rada bio je primena kompjuterske metodologije za procenu pozitivnosti Ki-67 antigena kod „adenomu sličnih“ i sa displazijom udruženih lezija ili masa (dysplasia associated lesions or masses - DALMs) i karcinoma kod ulceroznog kolitisa (UK) i da utvrdi novi pristup stepenovanju intenziteta bojenja Ki-67. Metode. Immunohistohemski preparati su analizirani da bi se proceni procent i intenzitet Ki-67 pozitivno obojenih čelija kod ukupno 50 „adenomu sličnih“ DALMs (27 sa displazijom niskog stepena i 23 sa displazijom visokog stepena) i 17 adenokarcinoma povezanih sa UK. Na osnovu automatske klasifikacije optičkih jestina jedara, određena su četiri stepen intenziteta imunohistohemskog bojenja. Rezultati. Indeks obeležavanja Ki-67 (labeling index – LI) kod displazije niskog stepena bio je značajno niži nego kod displazije visokog stepena i karcinoma (p < 0,001). Kod karcinoma Ki-67 LI nije se značajno razlikovao od vrednosti dobijene kod displazije visokog stepena (p > 0,05), međutim, postoje razlike u procentnim vrednostima kod umerno obojenih jedara (p < 0,05). Ukupne procente vrednosti hromogene optičke jestine jedara pokazale su statistički značajne razlike između DALMs i karcinoma (p < 0,05), mada ne i između normalne mukoze i displazije niskog stepena (p > 0,05). Zaključak. Dobijeni rezultati, prema ukupnom procenom obeleženih jedara, ukazuju na veliku sličnost između displazije visokog stepena i karcinoma, ali i da postoje razlike u procenatu umerno obojenih jedara. Pokazali smo da Ki-67 pozitivnost ima različitu unutrašnju distribuciju koja može da bude korisna u analizi ovih lezija. Ovi nalazi, takođe, ukazuju na važne bioškolske razlike između displazije niskog stepena i karcinoma kod UK i niski proliferativni potencijal displazije niskog stepena. Automatska analiza slike omogućuje objektivnu procenu Ki-67 imunohistohemskog bojenja kod displazija i karcinoma povezanih sa UK.

Ključne reči: kolitis, ulcercativni; polipi; karcinomi; kompjuteri; imunohistohemija; Ki-67 antigen.
Introduction

Patients with long-standing extensive ulcerative colitis (UC) are at increased risk for the development of colorectal carcinoma1–4. Widely accepted classical risk factors are long disease duration and involvement of the entire colon1. This increased risk begins approximately 7–10 years following disease onset, and increases at a rate of 0.5–1.0% per year5. The risk of colorectal cancer (CRC) is much greater in the UC patients who also have primary sclerosing cholangitis, and family history of colon cancer6–7. Most studies reported that epithelial dysplasia is the most important marker of an increased risk of malignancy in patients with UC. Dysplasia in ulcerative colitis may be classified as flat and elevated dysplasia associated lesion or mass [DALMs])3. They are frequently associated with a high proportion of colorectal carcinoma and are an indication for colectomy3,4. However, recent data suggest that DALMs are a heterogeneous population of lesions, and that not all subtypes carry a high risk of malignancy8,9.

Dysplasia associated lesion or masses are subdivided into “non-adenoma-like” and “adenoma-like” lesions based on their endoscopic appearance10–12.

“Non-adenoma-like” DALMs were defined as elevated dysplastic lesions that were irregular, broad based, or structured lesion. Patients with a “non-adenoma-like” DALMs (regardless of the dysplasia grade), should be treated with colectomy because of the high probability of adenocarcinoma11.

“Adenoma-like” DALM was defined as a discrete, well-defined, sessile or peduncular polyp in which endoscopic and histopathological distinction from a sporadic adenoma was not possible10. Recent studies have highlighted the possibility of performing a colonoscopic polypectomy for patients with “adenoma-like” DALM12–14.

Dysplastic epithelium in UC is subdivided into low-grade and high-grade depending on the severity of the atypia2. There is a general consensus that the low-grade dysplasia warrants, at least requires, long-term colonoscopic surveillance, whereas the emergency of high-grade dysplasia is best managed by colectomy to eliminate potential strong risk of cancer development2.

Although hematoxylin and eosin (H&E) histopathology is still the “gold standard” for the grading of dysplasia and its distinction from regenerative epithelial changes, the previous study has revealed variations in the interobserver agreement in diagnosis and dysplasia grading in UC. Under the circumstances, there is the necessity for a precise estimation of cell proliferation deregulation in dysplasias, especially for objectivization of diagnostic processes, and finally for establishing new promising therapeutic approaches. One of the most reliable markers for cellular proliferation is monoclonal antibody Ki-67 (MIB-1)15.

The Ki-67, a large nuclear protein, probably plays an important role in the regulation of cell proliferation. Antigen is Ki-67 expressed during all active phases of the cell cycle (G1, S, G2, and M, grade, stage and metastasis), but not in the G0 phase. The expression increases with the cell progression rising during the second half of the S-phase and reaching a peak in the G2 and M-phase15. Immunohistochemical estimation of Ki-67 was previously used in dysplastic lesion associated with UC16–18. These reports determined the percentage, and/or location of Ki-67 staining, however none of them presented exact quantification of the targeted protein staining intensity.

In recent years, many efforts have been done to replace conventional estimation of immunohistochemically evidenced proteins in tumor nuclei. These attempts were based on separation and photometrical analysis of specific color of immunohistochemical chromogene, especially, diaminobenzidine (DAB)19–21. The problem of quantitative immunohistochemistry is accurate separation of chromogene stain from color of colocalized counterstain. The breakthrough, in this field was made by Ruifrok and Johnston, which proposed the color deconvolution algorithm, with which is possible to separate immunostained and counterstained components, even in cases of overlapping spectral absorption spectra, as well as colocalisation22. The major obstacle in immunoreactivity estimation is determining, as previously reported by Walker23, “what is positive” in tissue sample, in other words which level of visible chromogene is appropriate to determine if the target structure is positive or not.

The aim of this study, therefore, was to apply computer-assisted methodology in assessment of Ki-67 positivity in “adenoma-like” DALMs and carcinoma in chronic UC, as well as to determine a new approach to scoring their patterns.

Methods

The formalin-fixed paraffin-embedded tissue specimens from 50 polypectomy or colectomy resected “adenoma-like” DALMs (47 patients) and 17 resected adenocarcinomas (17 patients) were selected from the files of the Institute of Pathology, during the period from January 2000 to December 2006. All the patients had a history of long-standing (> 10 years) subtotal and total UC and their lesions had arisen from UC affected colonic mucosa.

The patients with DALM consisted of 31 males and 16 females (mean age: 52 years, range: 38–75 years). The carcinoma study group consisted of 12 males and 5 females with the mean age of 64 years (range: 45–79 years).

The histological criteria for UC-associated lesions, described by Riddell et al., were applied. Of the 50 “adenoma-like” DALMs evaluated, low-grade dysplasia (LGD) was in 27, and high-grade dysplasia (HGD) in 23 specimens. All tissue samples were independently evaluated by the two pathologists. When a discrepancy in dysplasia grading occurred between the two observers, the lower of the values was used as the final one.

For the positive controls, 30 specimens of normal colonic mucosa from 23 surgically resected samples, sufficiently distant from sporadic CRC, were obtained.

Multiple serial 4 μm tissue section were prepared, one of which was stained with H&E for histological evaluation, and the others were used for immunohistochemistry.

Immunohistochemistry for Ki-67

The slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes at room temperature and washed in PBS for 5 minutes. Antigen retrieval was performed by heating the sections for 10 minutes at 100 °C in 10 mM citrate buffer (pH 6.0), and the left to cool to room temperature for 20 minutes. The primary antibody, anti-Ki-67 (MIB-1, Dako Corporation; Glostrup, Denmark) was incubated with the sections at 1:100 dilution for 18 hours at 4 °C. The sections were thoroughly rinsed and exposed to the secondary antibody (Dako) for 30 min at room temperature. Staining was performed with the Labeled Strept-Avidin Biotin (LSAB) method, using 3,3′-diaminobenzidine as chromogen. The sections were counterstained with 0.1% hematoxylin (Sigma), dehydrated, and mounted.

The negative controls were incubated in buffer without a primary antibody.

Image acquisition and analysis

The microscopic slides of all the groups stained for MIB-1 were examined on microscope Leica DMR (Leica Microsystems, Reuil-Malmaison, France), at the overall magnification of 200x. Bright field microscopic images (five the most representative areas from each slide, containing at least 1 000 nuclei per case) were obtained under the same conditions (intensity of light, aperture of condensor system), white-balanced and captured as digital images (8-bit RGB color images, 2088 × 1552 pixels) using digital camera (Leica DC 300). The files were saved as uncompressed TIFF format of the sizes about 9 MB. From each image, taking the whole glands in account, more than 1000 nuclei per case were interactively selected using Genius tablet (EasyPen, KYE Systems Corp.), and Adobe Photoshop’s v. 7.0 function polygonal lasso (adding new nuclei to multiple selection constantly holding shift key), cut, pasted, and merged in new generated image with white background (with the same pixel dimensions), which was saved as a separate file. Separation of the image files into blue (hematoxylin-image), and brown (DAB-image) was performed using colour deconvolution plugin by Landini, written for image analysis software Scion Image v. 1.37a for Windows (Scion Corporation, Frederick, MD, U.S.A.), while the images with chromogene were only saved as separate files. The Olympus MicroImage Software, v. 4.0 for Windows (Media Cybernetics, Silver Spring, MD, U.S.A) was used to determine outlines of nuclei by applying histogram based threshold on previously prepared, non-deconvoluted images with white background, which were finally saved as separate file of settings. Saved outlines were then applied on deconvoluted images (with chromogene only), to calculate the percentage of labeled nuclei, and mean optical density, per each analyzed case. Optical density represented the strength of pixel’s brown signal on images, and pixels which were stained blue prior to deconvolution had the value of 0. The four grades of immunohistochemical staining intensity (0, 1+, 2+, and 3+) were established by MicroImage’s autoclassification according to the iteration of nuclear optical densities. Grade 0 was considered immunohistochemically negative for Ki-67 staining, grade 1+ weak, 2+ moderate, and 3+ strong intensity.

Statistical analysis

Statistical evaluation was carried out with the software SigmaStat for Windows v 3.0 (SPSS Inc, Chicago, IL, USA). The differences between the groups and grades of the examined cases were assessed by Mann-Whitney test. A \( p \)-value < 0.05 was considered significant.

Results

The immunohistochemical patterns showed a great diversity in the crypts of the examined cases. In DALMs with low-grade dysplasia, Ki-67 immunoreactivity was prominent mainly in the expanded basal zone of the crypt (Figure 1), and rarely evident in the surface epithelium. However, in cases with high-grade dysplasia, Ki-67 was diffusely distributed throughout the crypts (Figure 2). In the normal colonic mucosa, only the cells of the crypt base were labeled. Diffuse Ki-67 immunoreactivity was observed in irregular, back to back and markedly budded crypts of carcinomas (Figure 3).
The great heterogeneity of staining intensity with MIB-1 was obvious in each of the examined groups. The staining pattern of nuclei differed from the diffuse weak intensity, moderate granular, to homogenous strong.

According to quantitative immunohistochemistry, the percentage of Ki-67 negative nuclei was the highest in the group of normal mucosa (76.86±4.99), and the lowest in group of carcinomas (21.47±10.88). Weak (1+) positive cells showed a statistically significant difference among normal colonic mucosa and low-grade dysplasia (p < 0.001), and low-grade dysplasia and high-grade dysplasia (p < 0.05). However, the differences between high-grade dysplasia and carcinoma were not significant (p > 0.05). Moderate (2+) positive cells presented significant differences of labeling indices among all groups, with the higher percentage of Ki-67 positive cells in carcinomas (30.49±2.73). In (3+) strong positive cells no significant differences were noted only between high-grade dysplasia and carcinoma (p > 0.05) (Table 1).

The differences in optical density values between normal mucosa and low-grade dysplasia in each intensity grade were not found (p > 0.05). Optical density, raised with grade of staining intensity, and showed the highest values in the grade 3+ of carcinomas (0.208±0.007). Statistically significant differences (p < 0.05; p < 0.001) were noticed between low-grade dysplasia, high-grade dysplasia and carcinoma of the grades 1+, 2+, and 3+ (Table 1).

As shown in Table 2, the overall percentage of Ki-67 positive cells was statistically different between examined histology groups (p < 0.001), except between high-grade dysplasia and carcinoma (p > 0.05).

### Table 1

Comparative survey of the labeled nuclei percentage, and optical density according to different grades of Ki-67 staining intensity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Grades of Ki-67 intensity</th>
<th>Labeled nuclei percentage</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean ± SD</td>
<td>p &lt;</td>
</tr>
<tr>
<td>N</td>
<td>negative - 0</td>
<td>30</td>
<td>76.86±4.99</td>
</tr>
<tr>
<td>LGD</td>
<td>weak - 1+</td>
<td>27</td>
<td>51.42±5.52</td>
</tr>
<tr>
<td>HGĐ</td>
<td>moderate - 2+</td>
<td>23</td>
<td>31.30±7.14</td>
</tr>
<tr>
<td>CRC</td>
<td>strong - 3+</td>
<td>17</td>
<td>21.47±10.88</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>30</td>
<td>12.97±3.73</td>
</tr>
<tr>
<td>LGD</td>
<td>weak - 1+</td>
<td>27</td>
<td>18.60±0.75</td>
</tr>
<tr>
<td>HGĐ</td>
<td>moderate - 2+</td>
<td>23</td>
<td>20.52±1.29</td>
</tr>
<tr>
<td>CRC</td>
<td>strong - 3+</td>
<td>17</td>
<td>24.81±5.56</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>30</td>
<td>5.48±2.02</td>
</tr>
<tr>
<td>LGD</td>
<td>moderate - 2+</td>
<td>27</td>
<td>14.65±2.16</td>
</tr>
<tr>
<td>HGĐ</td>
<td>strong - 3+</td>
<td>23</td>
<td>28.95±4.78</td>
</tr>
<tr>
<td>CRC</td>
<td></td>
<td>17</td>
<td>30.49±2.73</td>
</tr>
</tbody>
</table>

N – normal; LGD – low grade dysplasia; HGĐ – high grade dysplasia, CRC – colorectal cancer

### Table 2

Overall percentage, and optical density of Ki-67 positive nuclei in relation to the groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Overall percentage</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD</td>
<td>p &lt;</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>23.15±4.99</td>
</tr>
<tr>
<td>LGD</td>
<td>27</td>
<td>48.58±5.52</td>
</tr>
<tr>
<td>HGĐ</td>
<td>23</td>
<td>68.70±7.14</td>
</tr>
<tr>
<td>CRC</td>
<td>17</td>
<td>78.53±10.88</td>
</tr>
</tbody>
</table>

N – normal; LGD – low grade dysplasia; HGĐ – high grade dysplasia; CRC – colorectal cancer
According to optical density of Ki-67 positive nuclei, statistical analysis showed that histology groups differed significantly ($p < 0.05$; $p < 0.001$), except for normal mucosa and low-grade dysplasia ($p > 0.05$) (Table 2). The examples of Ki-67 optical density in normal mucosa, low- and high-grade dysplasia and in carcinoma are shown in Table 2.

### Discussion

It is postulated that UC cancer is derived presumably from the fields of dysplastic precursor lesions. The concept of DALM, one subtype of dysplasia in UC, was first given by Blackstone et al. in 1981. Dysplasia associated lesion or masses are frequently associated with UC cancer 3-4. However, recent studies point out that not all subtypes ("adenoma-like DALMs") are at high risk of carcinoma 5,6,9,12-14.

Only a few reports have evaluated immunohistochemically Ki-67 protein in UC-associated dysplasia with the purpose of determining characteristics of cell proliferation, and its malignant potential 16-18, 25-26. Noffsinger et al. elucidated that in some dysplasias, Ki-67 is diffusely distributed throughout the crypts, suggesting complete deregulation of normal cell proliferation. Two other studies used the MIB-1 antibody to detect the percentage Ki-67 stained nuclei (labeling index, LI) in relation to the distribution throughout the mucosal crypts in UC-associated dysplasia 17, 18.

In this study, the percentage of Ki-67 positive cells varied considerably among the studied groups. Statistically significant differences in the percentage of Ki-67 positive cells occurred among carcinoma, low-grade dysplasia, and normal mucosa ($p < 0.001$). The percentage of Ki-67 positive cells was significantly higher in high-grade than in low-grade dysplasia and normal mucosa ($p < 0.001$). Our results showed a progressive increase in the Ki-67 LI in "adenoma-like" DALMs in the order of normal mucosa, DALM with low-grade dysplasia and DALM with high-grade dysplasia. The interesting observation was that Ki-67 LI of carcinomas was not significantly different from the value obtained for DALMs with high-grade dysplasia ($p > 0.05$). The finding related to overall percentage of positive cells is only partially concordant with the previously published results, which were based on a semiquantitative estimation of immunohistochemical labeling 17, 18. Andersen et al. did not find significant differences in the percentage of Ki-67 positive cells between low-grade dysplasia and high-grade dysplasia, as well as high-grade dysplasia and carcinoma, but they could show significant differences between carcinoma and low-grade dysplasia. However, Kullmann et al. found that Ki-67 LI is significantly higher in high-grade dysplasia compared with low-grade dysplasia. Routine estimation of immunohistochemical staining intensity, especially for the purpose of scoring different histopathological patterns, is substantially based on subjective determination of an average presence of the visible chromogene 16-18, 25, 26. In fact, "positivity" of immunohistochemically stained tissues is not a fully defined category, and represents more than an average value of staining intensity 23. In our results, according to an overall percentage of labeled nuclei, high-grade dysplasia is very close to carcinoma, however showing the difference in the percentage values of the grades of moderate stained nuclei. We showed that Ki-67 immunohistochemical "positivity" have different internal distribution which could be useful in analysing these lesions. One of the interesting findings is that the overall average values of optical density, showed no statistically significant differences between the normal mucosa and low-grade dysplasia, as well as in the different grades of staining intensity, which could be a reflection of similar Ki-67 protein amounts, and lower proliferative potential.

### Conclusion

Our method for quantification of MIB-1 expression is an eclectic solution based on earlier attempts to separate immunohistochemically labeled areas, separating colors, and calculating their photometrical properties 19-21, 24, 27. Moreover, we recommend a system capable to make a distinction between the different proliferative properties of "adenoma-like" DALM, and carcinoma in ulcerative colitis, minimizing a subjective estimation of their patterns.

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