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Proliferation and apoptosis in proliferative lesions of the colon and rectum

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Abstract Classically, neoplasia has been considered to be primarily a disturbance in the regulation of proliferation, but it is now clear that programmed cell death is dysregulated as well as proliferation. The genes that are implicated in the regulation of these processes, such as *p53*, *c-myc* and *bcl-2*, are often also altered in neoplasms. We have studied proliferation and programmed cell death in hyperplastic polyps, adenomas, carcinomas in adenomas and adenocarcinomas of the colorectum, using the MIB-1 antibody which recognizes the Ki-67 proliferation related antigen, and an in situ nick-end labelling procedure for histochemical labelling of proliferating and apoptotic cells. In addition, immunohistochemistry was used to study the expression of the p53, c-myc and bcl-2 proteins. The material studied consisted of 12 samples of normal mucosa, 8 hyperplastic polyps, 39 adenomas with different degrees of dysplasia and including 3 that carried a carcinoma, and 10 adenocarcinomas, all formalin fixed and paraffin embedded. The Ki-67 index indicated that proliferation increased progressively in hyperplasia, through different degrees of dysplasia in adenoma, to reach the highest level (Ki-67 index of 50%) in adenocarcinoma. Apoptosis also increased in hyperplastic polyps and in adenomas, but decreased significantly in adenocarcinomas. p53 Labelling was seen in 77% of the carcinomas but in only 3% of the adenomas. Expression of *c-myc* increased in adenomas and carcinomas. Furthermore, a shift from predominantly nuclear to predominantly cytoplasmic expression was seen in progressive neoplasms. Expression of *bcl-2* was increased in an occasional hyperplastic polyp, but was increased markedly in almost all adenomas. Strikingly, in the adenomas with a carcinoma, the carcinoma showed weaker *bcl-2* expression than the adenoma. In 20% of the carcinomas some bcl-2 staining was seen but this was less extensive than in the adenomas. Our findings indicate that in the progression from adenoma to carcinoma both increased proliferation and decreased apoptosis occur. This is paralleled by an increased expression of *p53* and an increased and predominantly cytoplasmic expression of *c-myc*, but a decreased expression of bcl-2. This decreased *bcl-2* expression does not lead to an increase in apoptotic activity.

Key words Apoptosis · Adenoma · Carcinoma · Genes · Lower digestive tract

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Introduction

Cancer has long been regarded as a disorder of proliferation. It has recently become clear that dysregulation of programmed cell death may be also involved in the pathogenesis of neoplasia. In general terms, the volume increase of a neoplastic cell mass will depend on the net result of cell production through cell division and cell loss through apoptosis. The relative contribution of increased cell division or decreased cell loss to the volume increase of a neoplasm is largely unknown; nor do we know whether this varies during the different phases of the process of carcinogenesis.

For the study of the balance between proliferation and apoptosis the adenoma–carcinoma sequence [1] in the colorectum provides an attractive model. These lesions occur frequently, are pathogenetically related and, although longitudinal follow-up studies to validate these hypotheses are difficult if not impossible to perform, progression from a premalignant to a malignant phase proceeds through a series of morphologically well-defined steps. Each of these steps has been fitted into a model for the molecular genetic sequence of events that results in colorectal cancer [2].

The involvement of the oncogene *K-ras* and of the tumour-suppressor genes *p53*, *APC* and *DCC* in colorectal carcinogenesis has been well established [3]. Of these genes, it is clear that *p53* is involved in both cell cycle and apoptosis regulation; the *p53* gene appears to be inactivated in up to 60% of colorectal carcinomas [4]. It has been shown that the p53 protein can cause cell cycle arrest but can also stimulate apoptosis, and the hypothesis currently favoured is that genomic damage leads to up-regulation of *p53* expression, which halts the cell cycle to allow DNA repair. When DNA damage is irreparable, apoptosis is induced by p53 [5].

Other regulators of proliferation and apoptosis are *c-myc* and *bcl-2* [6, 7]. Elevated levels of *c-myc* occur in up to 80% of colorectal cancers [8]. The myc protein is primarily involved in cell cycle progression, and in the absence of key growth factors *c-myc* induces apoptosis. *Bcl-2* expression is upregulated in most colorectal adenomas and also in a proportion of colorectal carcinomas [9, 10]. It is a member of a complex family of apoptosis-regulating genes [11, 12], which, when up-regulated, prolong cell survival through inhibition of apoptosis [13]. *Bcl-2* and *c-myc* have been assumed to cooperate in colorectal carcinogenesis [14].

Analysis of proliferation and apoptosis at the tissue level has become feasible with the development of tools that allow visualization of these events in individual cells. Proliferation can be visualized through immunohistochemical staining of nuclear antigens that are expressed exclusively in cycling cells [15]. Apoptosis can be visualized using nick end-labelling, which detects the characteristic internucleosomal DNA strand breaks [16]. In this paper we report on the proliferative and apoptotic index of normal colorectal mucosa, hyperplastic lesions, adenomas with varying levels of dysplasia and carcinomas (partly in adenomas) of the colorectum. We have also examined the expression of *p53*, *bcl-2* and *c-myc*, genes that play a role in the regulation of proliferation and apoptosis.

Materials and methods

A series of consecutive cases (concerning 57 lesions from 51 patients) of hyperplastic polyps (8), adenomas (39) and carcinomas (10) of the colorectum was retrieved from the files of the Department of Pathology, University Hospital, Rotterdam. From all carcinoma specimens, normal mucosa from the proximal or distal margins of resection, which was always more than 5 cm from the tumour, was taken as control tissue. All specimens had been fixed in 4% buffered formaldehyde and embedded in paraffin. Histological diagnoses were made on routine H&E stained tissue sections, using standard criteria. Dysplasia in adenomas (15 tubular, 11 tubulovillous, 13 villous) was graded into low (31) and high (8). In 3 adenomas a carcinoma was found.

Expression of p53, the c-myc protein and the Ki-67 proliferation-associated protein was determined on 5-μm paraffin sections. After mounting on APES (aminopropyltriethoxysilane, Sigma, St. Louis, Mo.)-coated slides, the sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by incubation (15 min, RT) in methanol with 3% H_2O_2. Antigen retrieval was performed for all three antigens by microwave treatment (2 cycles of 5 min at 700 W in a Biorad H2500 oven, in a 0.01 M citrate buffer, pH 6.0). After cooling (15 min), washing in phosphate buffered saline (PBS, pH 7.0) and preincubation in 10% normal goat serum (Dako, Glostrup, Denmark, 15 min), the sections were incubated with the primary antibody overnight at 4°C. The anti-p53 was a mouse monoclonal antibody, clone DO7 (Dako), used at a dilution of 1:50; the anti-Ki-67 was the MIB-1 mouse monoclonal antibody (Immunotech, Hamburg, Germany) used at a dilution of 1:100; the anti-c-myc was the clone GE10 antibody, raised against the C-terminal peptide of the human c-myc protein (Oncogene, New York, N.Y.), diluted 1:3000, the anti-bcl-2 was clone 124 (Dako) diluted 1:50.

After washing in PBS, bound antibodies were visualized using biotinylated rabbit-anti-mouse Ig (Dako) and the streptavidin–biotin-peroxidase system (Dako). Peroxidase activity was developed with 3–3' diaminobenzidine-HCl. The slides were lightly counterstained with haematoxylin and mounted.

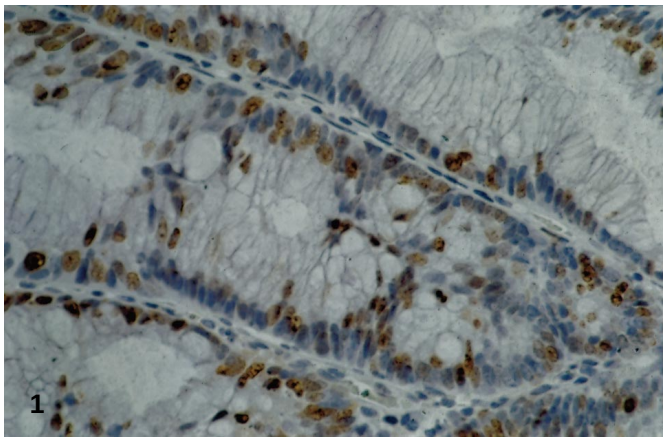
Apoptosis was visualized through detection of internucleosomal fragmentation of DNA, which was visualized using in situ nick-end labelling with terminal deoxynucleotidyl transferase (TdT; TUNEL) [16]. To this end, rehydrated sections were treated with proteinase K (20 μg/ml in 10 mM Tris-HCl buffer, pH 7.4; 15 min, RT). After washing, the sections were incubated with TdT (10 U/μl; Gibco) and 0.05 nmol/ul of biotin-16-dUTP (Boehringer, Mannheim, Germany), 2 h at 37°C. Subsequently the sections were washed in a citrate–saline buffer and incubated with a streptavidin–biotin peroxidase complex (Dako). As positive control, tissue sections were pre-treated (60 min) with 1 μg/ml DNA-seI (Stratagene, USA). Tissue sections incubated without TdT were used as negative control. Visualization of peroxidase activity was as for immunohistochemistry.

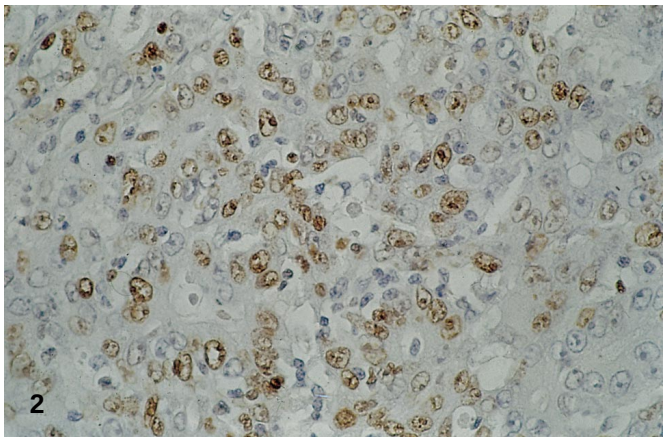
For counting, normal crypts were divided in three sections with approximately equal cell numbers. For normal crypts the Ki-67 and TUNEL index were determined by counting 200 and 2000 cells respectively in consecutive crypts. For tumours the Ki-67 and TUNEL indices were determined by counting 200 and 2000 cells in consecutive fields, chosen randomly in non-necrotic areas of the tumours. p53 Staining was regarded as positive when more than 5% of the nuclei showed intense staining. For c-myc, nuclear and cytoplasmic staining were semiquantitatively graded (negative, weak, moderate or strong). For bcl-2 staining grading into negative, focal or positive was used. Student's *t*-test for independent samples was used to assess the significance of the observed differences. Correlations were analysed using Pearson's correlation coefficient. Statistical significance was taken as $P<0.05$.

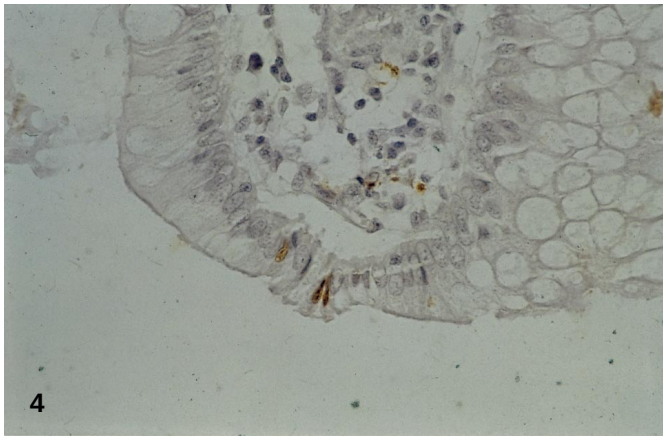
Results

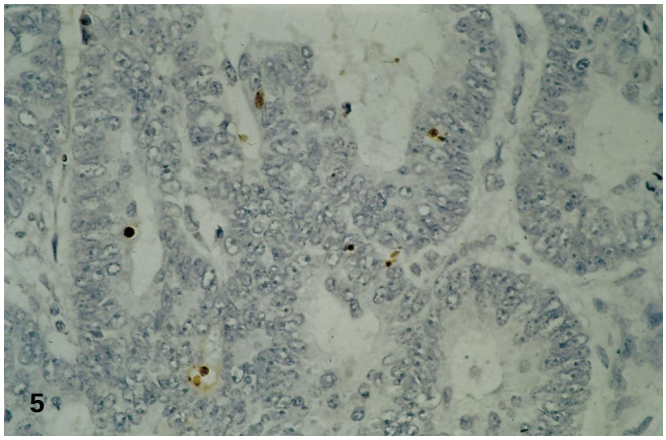
Of the 39 adenomas, 31 showed low-grade dysplasia and 8, high-grade dysplasia. In 3 adenomas an intramucosal carcinoma was identified. Of the 10 carcinomas, 3 were well differentiated and 7 moderately well differentiated.

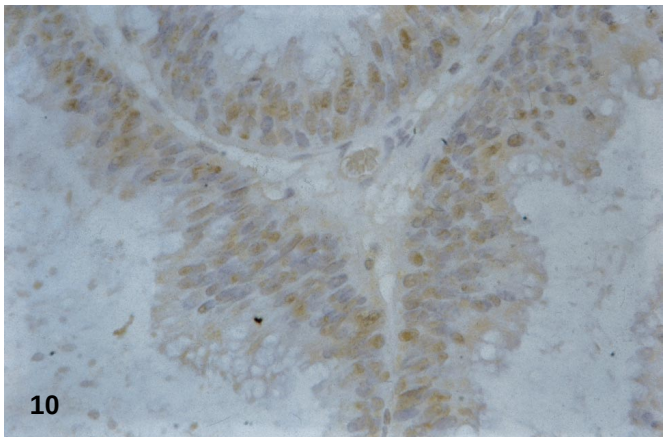
The Ki-67 labelling is illustrated in Figs. 1 and 2, and the labelling indices in the analysed tissue specimens are shown in Fig. 3. As expected, the Ki-67 labelling index decreased from the crypt base (27.7 ± 11.1%) to the luminal surface (0.1 ± 0.1%). In hyperplastic polyps the mean Ki-67 index (24.5 ± 4.8%) was comparable to that in the crypt base. Proliferating cells were mainly confined to the lower half of the crypt. In adenomas the Ki-67 index increased significantly throughout the lesion and in correlation with the degree of dysplasia (26.5 ± 8.8% in low-grade vs 34.8 ± 5.8% in high-grade adenomas, $P<0.05$). In carcinomas the highest labelling index (53.1 ± 10.5%) was obtained ($P<0.001$ vs any other category).











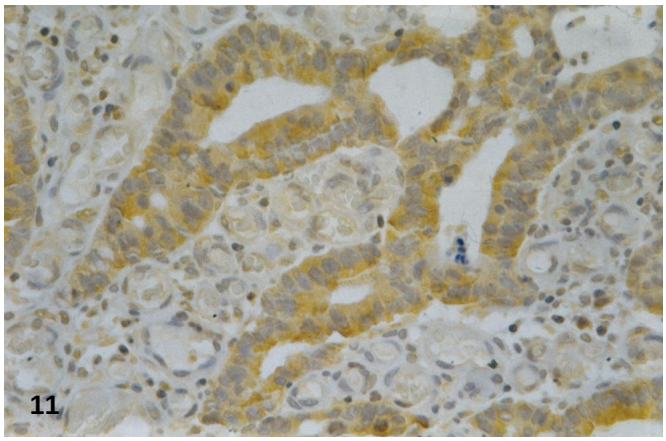


Fig. 1 Ki-67 labelling of an adenoma. Proliferating cells are not limited to a proliferation zone but are dispersed throughout the lesion. ×200

Fig. 2 Ki-67 labelling of a carcinoma. ×200

Fig. 4 TUNEL labelling of normal colon mucosa. Labelling is limited to cells at the mucosal surface. ×200

Fig. 5 TUNEL labelling of an adenocarcinoma. ×200

Fig. 10 Immunoperoxidase staining for c-myc in an adenoma. ×200

Fig. 11 Immunoperoxidase staining for c-myc in a carcinoma. Note that in the adenoma (Fig. 6) the staining is predominantly nuclear, whereas in the carcinoma predominantly strong cytoplasmic staining is observed. ×200

The TUNEL labelling is illustrated in Figs. 4 and 5, and the labelling indices of the analysed specimens are shown in Fig. 6. The TUNEL index in the normal crypt increased (from 0.4 ± 0.4% to 2.6 ± 1.3%) towards the crypt surface. In hyperplastic polyps TUNEL staining extended downward into the crypts. The TUNEL index corresponded to that of the mucosal surface. Apoptosis occurred throughout the lesion in all neoplasms. The TUNEL index was not higher in low-grade adenomas (3.9 ± 2.1%), but was significantly higher in high-grade adenomas (5.6 ± 3.4%, $P<0.05$). In carcinomas the TUNEL index was lower (3.1 ± 1.2%) than in high-grade adenomas, but this difference had only borderline significance. In adenomas, a trend towards a positive correla-

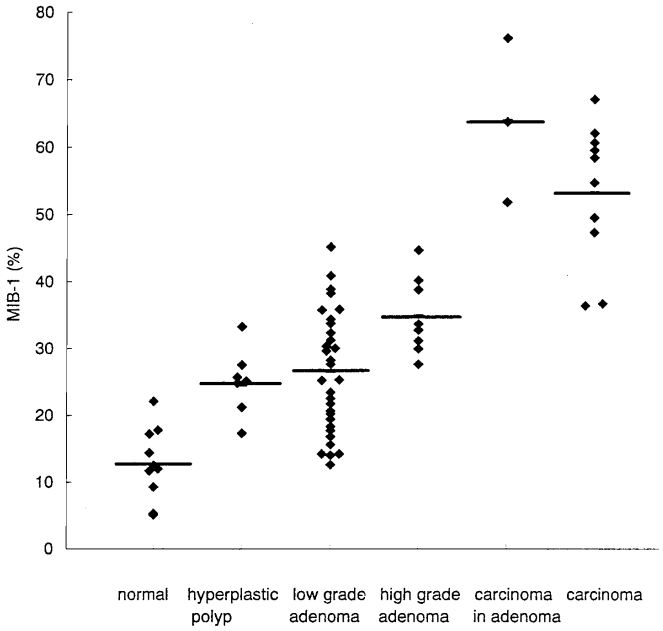


Fig. 3 Ki-67 index in normal mucosa and in hyperplastic and neoplastic lesions of the colorectum. *Horizontal bars* indicate the mean (normal versus all lesions $P<0.001$; low-grade and high-grade adenomas vs carcinomas $P<0.001$)

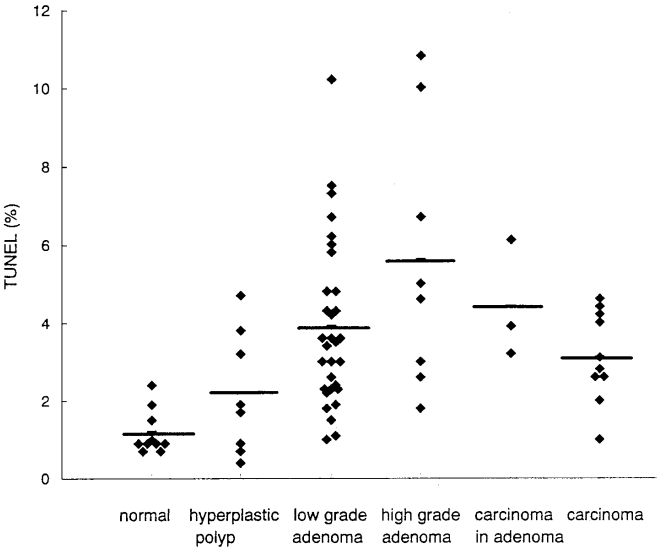


Fig. 6 TUNEL index in normal mucosa and in hyperplastic and neoplastic lesions of the colorectum. *Horizontal bars* indicate the mean (normal vs all adenomas $P<0.001$; normal vs all carcinomas $P<0.001$; high-grade adenomas vs all carcinomas $P = 0.07$)

tion ($P = 0.07$) was found between the Ki-67 and TUNEL labelling indices (Fig. 7). For the group of carcinomas no such trend was apparent (data not shown).

Nuclear p53 immunohistochemical staining was found in 6 of 10 (60%) of the carcinomas as well as in all 3 carcinomas in adenoma (which makes 9 out of 13 carcinomas, 77%, positive), but in only 1 (high-grade dysplasia) adenoma. Neither the Ki-67 nor the TUNEL

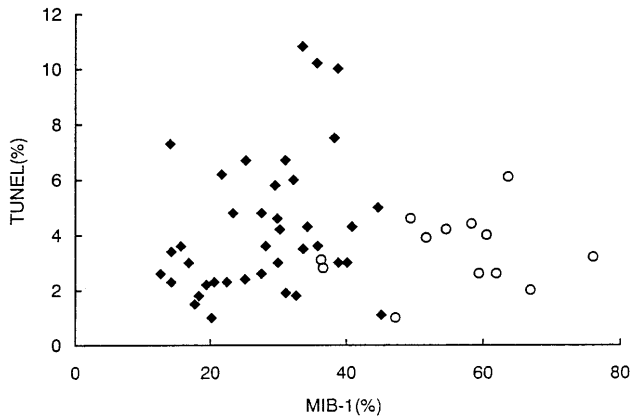


Fig. 7 Relationship between Ki-67 and TUNEL indices in adenomas (◆) and carcinomas (○). For adenomas $P = 0.07$; $R = 0.29$

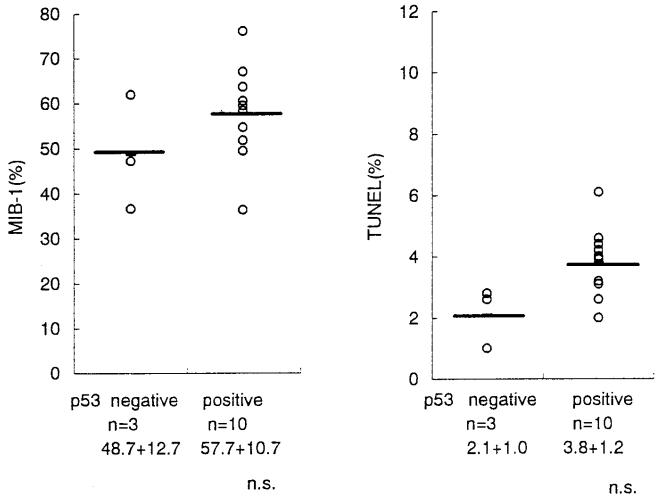


Fig. 8 p53 Staining in relation to Ki-67 and TUNEL index in colorectal carcinomas. No significant differences are observed

index was significantly correlated with p53 expression (Fig. 8), although both tended to be higher in p53-positive cases. Expression of p53 was not found in hyperplastic polyps or normal mucosa.

As reported previously [17], we found bcl-2 staining in normal crypt base cells, which appeared to increase in 3 (37%) hyperplastic polyps. Of the adenomas, 85% were positive either focally or diffusely without any significant difference according to the degree of dysplasia. Of all carcinomas (including the 3 carcinomas in adenoma) 5 (40%) stained for bcl-2, although often weakly. Notably, the carcinomas in bcl-2 positive adenomas showed reduced staining. Bcl-2 staining correlated neither with the Ki-67 index nor with the TUNEL index (Fig. 9).

Moderate to strong staining for c-myc protein was found in 62% (24/39) of the adenomas and in 77% (10/13) of the carcinomas (including the carcinomas in adenoma; Figs. 10, 11). The staining pattern was predominantly nuclear in the adenomas. Moderate to strong

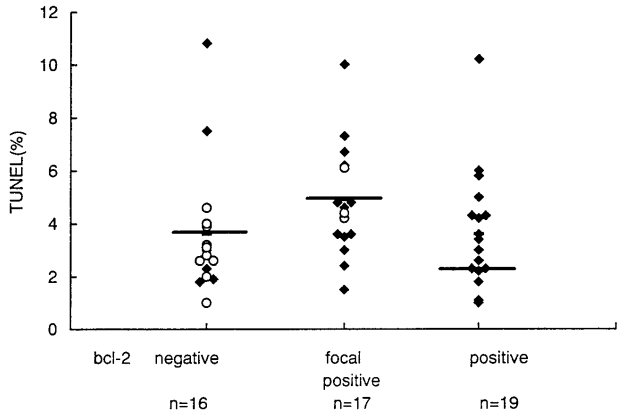


Fig. 9 Relationship between bcl-2 staining and TUNEL index in adenomas (◆) and carcinomas (○). The differences observed are not significant

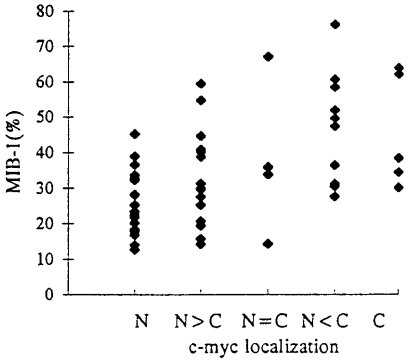


Fig. 12 Semiquantitative evaluation of staining for c-myc in relation to Ki-67 labelling. With increasing Ki-67 index c-myc staining shows a shift from predominantly nuclear to predominantly cytoplasmic ($P<0.001$; $R = 0.52$; *N* exclusively nuclear staining, *N>C* more nuclear than cytoplasmic staining, *N = C* equal nuclear and cytoplasmic staining, *N<C* more cytoplasmic than nuclear staining, *C* exclusively cytoplasmic staining

cytoplasmic c-myc staining was found in 33% (13/39) of the adenomas and in 77% (10/13) of the carcinomas. When all neoplasms were taken together a strong correlation between high predominantly cytoplasmic c-myc staining and Ki-67 index was found (Fig. 12). Nuclear c-myc staining correlated neither with the Ki-67 nor with the TUNEL index.

Discussion

Most colorectal carcinomas are assumed to develop from colorectal adenomas through gradual accumulation of multiple genetic defects [2]. Some of these involve genes whose product plays a role in tissue homeostasis. For a long time the emphasis in models of carcinogenesis has been concentrated almost exclusively on increased cell proliferation. It has become well established, however, that decreased apoptotic cell loss also plays a significant part [18, 19]. Remarkably, genes that have a role in the regulation of the cell cycle often also function in the regulation of apoptosis [5], with *p53* and *c-myc* as examples. Up-regulation of *p53* expression occurs subsequent to genome damage, which leads to cell cycle arrest to allow for DNA repair to take place. Irreparable damage, however, leads to *p53*-mediated apoptosis.

Expression of the c-myc protein is normally seen early in the G_1 phase of the cell cycle, and up-regulation of *c-myc* expression without a concurrent growth factor stimulus leads the cell to undergo apoptosis [18]. The regulation of apoptosis appears to be a complex process involving many genes; one of the first of these to be recognized as apoptosis regulator was *bcl-2*, which encodes for an apoptosis blocking protein [13]. It has subsequently been shown that *bcl-2* is member of a family of apoptosis regulators, which also includes *bax*, *bcl-x* and *bak* [11, 12]. The balance between cell production through proliferation and cell loss through apoptosis determines how fast a tumour grows and, consequently, the balance between regulators of proliferation and apoptosis is an important determinant of tumour behaviour. The aim of the present study was to document proliferative and apoptotic activity in benign, premalignant and malignant disorders of growth in the colorectum and to correlate these with the expression of regulators of apoptosis. To visualize proliferation and apoptosis we chose Ki-67 labelling and TUNEL, both of which are accepted standard techniques for this type of analysis. The reliability of our histochemical findings was assessed by comparing our results with those previously reported by other groups [19–22]. Our TUNEL labelling patterns of normal mucosa correspond closely to those of Sträter et al. [19], and proliferation and apoptosis indices correspond closely to those of Tsujitani et al. [20]. The indices reported by Baretton et al. [21] are somewhat lower, but these authors considered only TUNEL-positive cells that showed additional morphological features of apoptosis, which might account for the difference. We excluded necrotic areas from our TUNEL analyses to avoid over-estimation due to false positive labelling of necrotic cells.

As expected, apoptotic activity was inversely correlated with proliferative activity in the normal colon. Apoptotic cells were found almost exclusively on the luminal surface of the mucosa. The proliferative activity of hyperplastic polyps corresponded with that of the normal crypt base whereas the apoptotic activity corresponded with that of surface epithelial cells. Neither was limited to the crypt base but also occurred closer to the lumen. These findings indicate that for the development of hyperplastic polyps, a phase of increased proliferation may be responsible, which is almost but not completely balanced by increased apoptosis. The proliferative activity increased in adenomas with high-grade dysplasia; low-grade dysplastic adenomas displayed a proliferative activity that corresponded to that of hyperplastic polyps. A corresponding increase in apoptotic activity was found in colorectal adenomas. The highest proliferative activity occurred in the carcinomas. Strikingly, in the carcinomas a trend towards a reduction in apoptotic activity was found.

Bedi et al. [22] studied apoptosis in the adenoma–carcinoma sequence using a slightly different technique and also found apoptosis to be reduced in carcinomas. Tsujitani et al. [20] compared the carcinoma component with the adenoma component in carcinomas in adenoma and also found decreased apoptosis in the carcinomas in combination with increased proliferative activity. These findings, in line with earlier observations [23, 26], indicate that adenomas arise primarily through increased proliferative activity, which is only partly compensated for by the rate of apoptosis, though it does rise in adenomas. The presumably higher growth rate of carcinomas relative to adenomas may be partly due to a decreased rate of apoptosis. Tatebe et al. [27] recently investigated apoptosis and proliferation in primary and metastatic colon cancers. For primary carcinomas their findings correspond well with ours. An interesting observation in their study is an increased proliferation rate in lymph nodes and liver metastases, together with a relatively greater increase in apoptotic activity, suggesting a relatively higher cell loss and consequently a lower growth rate in metastatic tumours.

To clarify the role of apoptosis-regulating genes, we compared proliferation and apoptosis indices with the expression of p53, bcl-2 and c-myc. We found *p53* expression in 10 of 13 (77%) of the carcinomas (including the 3 carcinomas in adenoma), but in only 1 (3%) adenoma. In the carcinoma group, p53 and proliferation index were not correlated. There was a strong correlation ($P<0.0001$) when adenomas and carcinomas were pooled, but this can be accounted for by the low rate of p53 positivity and the generally lower proliferation index in adenomas. There was a trend for apoptosis to be increased in p53-positive carcinomas but this was not statistically significant. Bcl-2 expression in colorectal adenomas and carcinomas has been previously reported [9, 10, 14, 17, 28–31]. Hague et al. [28] and Sinicrope et al. [14] reported bcl-2 staining in over 60% of colorectal carcinomas. Our data, which have already been reported [17], are in agreement with those of Scott et al. [29] and Kaklamanis et al. [30] and indicate that relative to colorectal adenomas, colorectal carcinomas show a reduction in bcl-2 expression. Given the function of *bcl-2* as apoptosis suppressor and the decreased rate of apoptosis in colorectal carcinomas this paradox is not easy to explain. It has become clear, however, that the regulation of apoptosis is complex and involves not only *bcl-2* but also a variety of other members of this gene family [11, 12], in addition to other regulating genes, including *p53* and *c-myc*. This might imply that carcinomas acquire other genetic lesions, which render *bcl-2* redundant [29]. Bcl-2 expression was not correlated with p53 expression, as reported previously [29]. When adenomas and carcinomas were taken together, a strong negative correlation was found between proliferative activity and bcl-2 expression.

Our results regarding the expression of c-myc are similar to those of earlier reports [31, 32]. In normal and hyperplastic mucosa c-myc staining was limited to crypt base cells. Increased *c-myc* immunoreactivity was found in a combination of nuclear and cytoplasmic staining in 77% of the neoplasms. Staining was more frequently nuclear in adenomas but cytoplasmic in carcinomas. This probably accounts for the positive correlation we found between cytoplasmic c-myc staining and the proliferative index. No correlation was found between bcl-2 staining and c-myc staining. Earlier reports have documented increased c-myc staining with advanced Dukes stage and shortened disease-free survival and decreased *c-myc* expression with cellular differentiation [31, 32]. Overall, these data suggest that in the progression of colorectal neoplasms increased c-myc expression occurs with a preferentially cytoplasmic pattern. Although c-myc is a nuclear phosphoprotein, the cytoplasmic staining pattern in cancer is unlikely to be an artefact, because it can be produced with several antibodies, as has been reported repeatedly. As suggested by Mooy et al. [33], the cytoplasmic distribution might be due to (undefined) protein alterations.

In conclusion, in hyperplastic polyps of the colon and rectum an almost balanced increase in proliferation and apoptosis is found, with a mostly normal pattern of expression of the apoptosis-regulating genes *p53*, *bcl-2* and *c-myc*. In the adenoma–carcinoma sequence a continuous increase in proliferative activity is found, whereas apoptosis tends to decrease in carcinomas. A corresponding pattern of *bcl-2* expression indicates that *bcl-2* is not the sole regulator of apoptosis in colorectal neoplasms. In the adenoma–carcinoma sequence *c-myc* expression gradually increases with a shift towards a cytoplasmic pattern.

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