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Xingpei Hao · Mingqing Du · Anne E. Bishop
Ian C. Talbot

Imbalance between proliferation and apoptosis in the development of colorectal carcinoma

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Abstract To evaluate the relationship between cell proliferation and apoptosis in sporadic colorectal carcinogenesis, immunohistochemistry for proliferation-associated antigen Ki-67 and in situ end labelling for identifying apoptotic bodies were performed on paraffin sections from 59 adenomas and 22 carcinomas. These results were correlated with the expression of the proliferation and apoptosis modulators Bcl-2 and p53. Carcinomas showed increased proliferation and apoptosis compared with adenomas ($P<0.0001$, $P<0.001$, respectively). There were positive linear correlations between proliferation and apoptosis in adenomas and carcinomas ($P<0.02$, $P<0.05$, respectively). The proliferative rate increased significantly from mild to moderate, and from moderate to severe dysplasia ($P<0.002$, $P<0.001$, respectively). Apoptotic rate also increased in this sequence, but the increases did not reach statistical significance (both $P>0.05$). Expression of Bcl-2 was associated with lower apoptotic rate in adenomas ($P<0.025$) but not in carcinomas ($P>0.25$), whereas p53 expression was correlated with higher proliferative rate in both adenomas and carcinomas ($P<0.01$, $P<0.05$, respectively). An inverse relationship between Bcl-2 and p53 expression was seen in both adenomas and carcinomas ($P<0.05$, $P<0.005$, respectively). These data suggest that the normal balance between proliferation and apoptosis is disturbed in colorectal carcinogenesis, both being increased, but proliferation occurs in excess. Bcl-2 and p53 may each play a role in modulating cell apoptosis or proliferation during the development of colorectal carcinoma.

Key words Colorectal carcinogenesis · Apoptosis · Cell proliferation · Adenoma · Carcinoma

X. Hao · I.C. Talbot (✉)
Academic Department of Pathology,
ICRF Colorectal Cancer Unit, St Mark's Hospital,
Watford Road, Harrow HA1 3UJ, UK
e-mail: talboti@icrf.icnet.uk
Tel.: +44-0181-235-4220, Fax: +44-0181-235-4277

M. Du
Department of Histopathology,
University College London Medical School,
London WC1E 6JJ, UK

A.E. Bishop
Department of Histochemistry,
Imperial College School of Medicine, Hammersmith Hospital,
London W12 ONN, UK

Introduction

Renewal of normal epithelial tissues involves proliferation, migration, differentiation and apoptosis (programmed cell death) [1], which together maintain epithelial homeostasis. Perturbance of these processes results in neoplastic transformation and progression. It is accepted that the contribution of apoptosis to neoplastic development is as important as that of proliferation. Several gene products, such as p53 and Bcl-2, are involved in modulating cell proliferation as well as apoptosis and are interrelated [2]. For instance, wild-type p53 induces apoptosis in cells with irreversible DNA damage, while mutant p53 inhibits apoptosis and confers an advantage for cell survival [3, 4]. Mutant p53 has been shown to inhibit Bcl-2 expression in a mammary epithelial cell line [5]. Ectopic p53 in lymphoid cells was also shown to down-regulate endogenous Bcl-2 expression while simutaneously increasing the expression of Bax [6, 7]. On the other hand, p53-induced cell death can be prevented by Bcl-2 expression [8], and Bcl-2 expression in p53-deficient mice is enhanced, with accompanying down-regulation of Bax expression [6]. These results suggest that p53 and Bcl-2 may interact with each other and have different roles in oncogenesis. Therefore, gaining further insight into the relationship between cell proliferation and apoptosis and the factors that control these processes will help to shed light on the pathogenesis of tumours.

Colorectal carcinoma remains the second most frequent fatal cancer in the western world. Most arise from pre-existing adenomas. Cell kinetics studies suggest that the tumour growth rate is high in both animal and human colorectal adenomas [9, 10]. Since human colorectal adenomas may remain stationary for a long time before

transforming to malignancies [11], a large number of cells must be lost while growth is slow. To evaluate the role of proliferation and apoptosis in the development of sporadic colorectal carcinoma, 59 adenomas and 22 carcinomas were quantitatively analysed by immunohistochemistry for the proliferation-associated antigen Ki-67 and by in situ end labelling for apoptotic cells. Furthermore, expression of p53 and Bcl-2 was also examined in an attempt to elucidate the links with proliferation, apoptosis and their relative contributions to the development of colorectal cancer.

Materials and methods

Fifty-nine sporadic adenomas were selected from the files of the Academic Department of Pathology, St. Mark's Hospital, according to the following criteria: only patients without any previous history of malignancies or familial adenomatous polyposis were chosen; all polyps were completely excised by polypectomy or surgical resection; if multiple, the adenomas chosen were the largest and showed the most severe dysplasia. For comparison, 22 carcinomas were also examined. Serial sections were cut at 4 µm thickness from unbuffered formol–saline-fixed, paraffin-embedded blocks and placed on poly-*l*-lysine-coated (Sigma Chemicals, St. Louis, Mo.) slides. One section was stained with haematoxylin and eosin and used for histological classification, and the others were immunostained. The grading and staging of the tumours were based on the WHO classification system [12]. To ensure consistency in the grading of dysplasia, the H&E-stained sections were first read by one observer (X.P.H.) and the results were compared with those in the original histopathological reports. Any cases in which there was doubt about the grade of dysplasia were read again by an experienced histopathologist (I.C.T.), and an agreement was reached.

A standard ABC method was used for immunohistochemistry. Briefly, tissue sections were dewaxed in xylene and rehydrated through graded alcohols to distilled water, and then immersed in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to delete endogenous peroxidase. Subsequently, sections were subjected to antigen retrieval by boiling in sodium citrate buffer (0.01 M, pH 6.0) for 2 min in an aluminium pressure cooker at 15 psi. Following this, non-specific staining was blocked with normal horse serum for 10 min. Monoclonal antibody to Ki-67 antigen (1:50 in PBS [phosphate-buffered saline], Dako, isotope: IgG1, kappa) was applied to tissue sections and these were incubated in a humidified chamber overnight at room temperature. Following rinsing in PBS, the sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA 94010, USA, 1:200) for 30 min and sequentially stained using the Vectastain Elite ABC Kit (Vector Laboratories, CA 94010, USA, 1:50 for 30 min). 3-3' Diaminobenzidine (DAB; Sigma Chemicals, St. Louis, Mo.) was used as the chromogen. Formalin-fixed, paraffin-embedded sections of human breast carcinoma served as positive controls, while negative controls were obtained by using PBS in place of the primary antibody. Immunostaining of Bcl-2 and p53 was carried out as previously described [13].

In situ end labelling (ISEL) was employed with minor modifications to detect apoptotic cells [14]. Briefly, sections were dewaxed in xylene and rehydrated through graded alcohols to distilled water, and then incubated in 0.1% H_2O_2 in PBS for 30 min to block endogenous peroxidase. Sections were digested with proteinase K (5 µg/ml in 50 mM Tris-HCl, pH 8.0) at 37°C for 15 min, rinsed three times in distilled water, then once in 70%, 90% and 100% ethanol, and air-dried. A volume of 50 µl of labelling mixture containing 0.5 nmol biotin-11-dUTP (Sigma Chemicals, St. Louis, Mo.), 2.5 U of the Klenow fragment of DNA polymerase I (Sigma Chemicals), 0.01 mM each of dTTP, dCTP, dGTP in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 0.005% BSA was applied to each section and covered with a coverslip. Sections were incubated at 37°C for 2 h, and the reaction was terminated by washing in distilled water three times. Subsequently, sections were incubated with the Vectastain Elite ABC Kit (Vector Laboratories, CA 94010, USA, 1:50) for 30 min and washed in PBS. Sections were finally visualised in 3-3' diaminobenzidine, lightly counterstained with haematoxylin, and mounted. Paraffin-embedded sections of a normal tonsil were used as positive controls, while a negative control was obtained by omission of DNA polymerase from the labelling mixture. Since ISEL labels both apoptotic and necrotic cells, only those cells with characteristic morphological changes, such as cytoplasmic and nuclear condensation, were regarded as positively labelled apoptotic cells [15, 16]. Apoptotic fragments in glandular lumina were excluded from the analysis, as these were considered to be cell debris resulting from necrosis and inflammation.

At least 1000 cells were counted in each case by microscopic examination at ×400 magnification, and the proliferative and apoptotic indices were calculated as the percentage of total cells found to be positive. As the mean value of the proliferative index (PI) of adenomas and carcinomas was 30.5%, a value of 30% or less was designated as low and a value over 30% as high. Similarly, the apoptotic index (AI) of adenomas and carcinomas was 1.53%, and a cut-off value of 1.5% was therefore used to divide high and low values.

Statistical evaluation of quantitative data between the two groups was carried out using Student's *t*-test. The Chi-square test was used to assess the associations between parameters and pathological data. The Pearson correlation coefficient was used for the assessment of correlation between the parameters tested. $P<0.05$ was considered statistically significant.

Results

The proliferative and apoptotic indices (PI and AI) are summarised in Table 1. Both the PI and AI of carcinomas were significantly higher than those of adenomas ($P<0.0001$, $P<0.001$, respectively, Figs. 1, 2). The proliferative rate increased significantly from mild to moderate and from moderate to severe dysplasia ($P<0.002$, $P<0.001$, respectively), but there was no difference between severe dysplasia and carcinoma ($P>0.50$). The apoptotic rate also increased progressively from mild to moderate dysplasia, moderate to severe dysplasia and severe dysplasia to carcinoma, but the increases did not reach statistical significance ($P>0.05$, $P>0.05$, $P>0.10$, respectively, Figs. 3, 4).

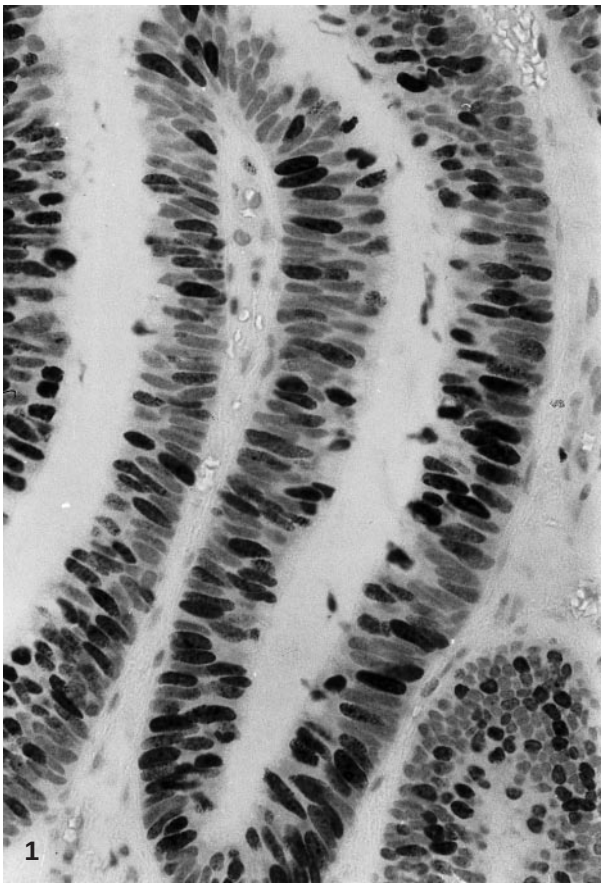
As shown in Tables 2 and 3, p53 nuclear accumulation was significantly associated with higher proliferative rates in both adenomas and carcinomas ($P<0.01$, $P<0.05$, respectively). Expression of Bcl-2 was inversely related to the lower apoptotic index in adenomas ($P<0.025$), but not in carcinomas ($P>0.25$). There was no correlation between p53 expression and the apoptotic index ($P=0.50$,

Fig. 1 Ki-67 expression in an adenomas with moderate dysplasia. Immunostaining, ×100 ▶

Fig. 2 Ki-67 expression in a well-differentiated carcinoma. Immunostaining, ×100

Fig. 3 Apoptotic bodies (*arrowed*) in an adenoma with moderate dysplasia. ISEL, ×160

Fig. 4 Apoptotic bodies (*arrowed*) in a moderately differentiated carcinoma. ISEL, ×160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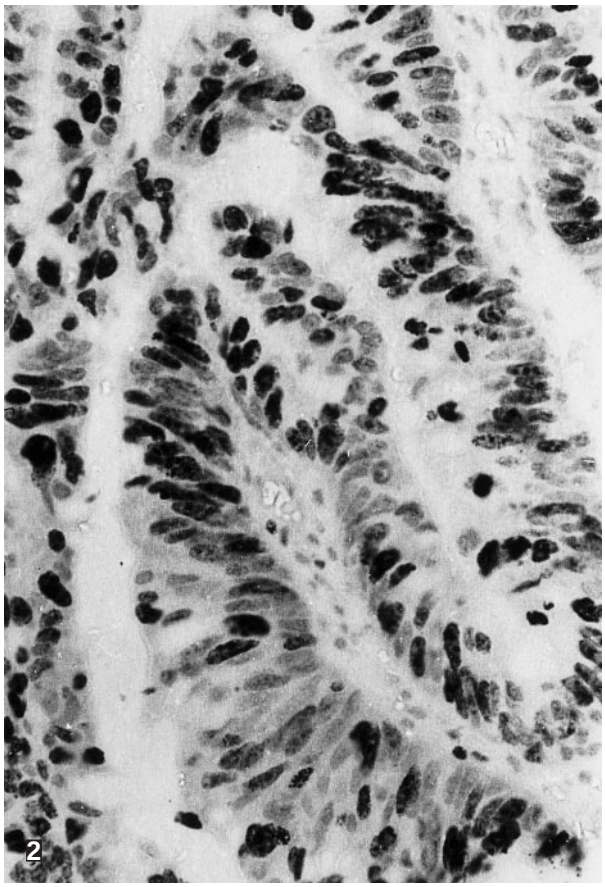
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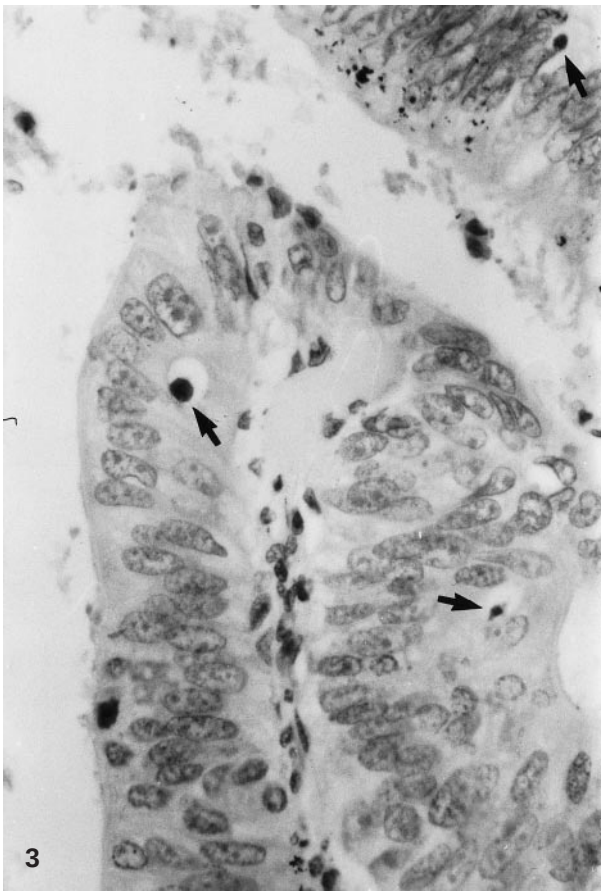
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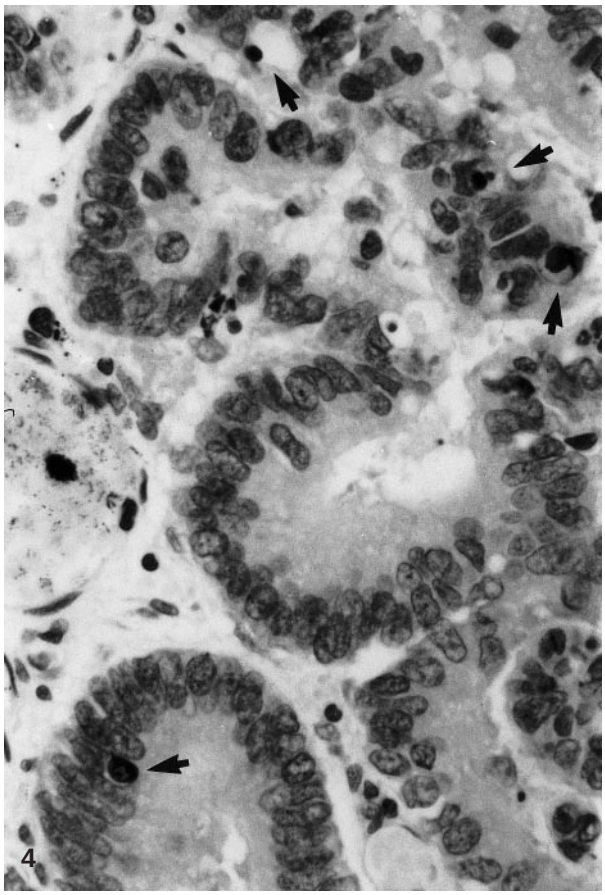
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Table 1 Proliferative (*PI*) and apoptotic indices (*AI*) in adenomatous and carcinomatous tissues

	n	PI	*P*	AI	*P*
Adenomas					
Dysplasia:					
Mild	21	16.6±4.64	<0.002[a]	1.08±0.65	>0.05[a]
Moderate	27	25.8±11.8	<0.001[b]	1.36±0.53	>0.05[b]
Severe	11	42.8±11.1	>0.50[c]	1.76±0.43	>0.10[c]
Total	59	25.69±13.0	<0.0001[d]	1.34±0.60	<0.001[d]
Carcinomas	22	43.35±10.28		2.02±0.82	

[a] Mild vs moderate
[b] Moderate vs severe
[c] Severe vs carcinoma
[d] Adenomas vs carcinomas

Table 2 Correlation between p53 expression and PI in colorectal adenomas and carcinomas

		p53			
		Adenomas		Carcinomas	
		+	–	+	–
PI	≤30%	6	32	3	7
	>30%	10	11	9	3
P		<0.01(χ^2=6.93)		<0.05 (χ^2=4.45)	

Table 3 Correlation between Bcl-2 expression and AI in colorectal adenomas and carcinomas

		Bcl-2				
		Adenomas		Carcinomas		
		High	Low	High	Low	–
AI	≤1.5%	36	11	1	2	4
	>1.5%	5	7	2	7	6

P <0.025 (χ^2=5.50) >0.25 (χ^2=0.56) (high + low) vs (–)

Table 4 Correlation among PI,AI, p53 and Bcl-2 expression in adenomas and carcinomas

	Adenomas			Carcinomas		
	n	*r*	*P*	*n*	*r*	*P*
Bcl-2 vs p53 (15)	73	–0.24	<0.05	54	–0.40	<0.005
AI vs PI	59	0.28	<0.02	22	0.44	<0.05

P>0.10 in adenomas and carcinomas, respectively; data not shown) or between Bcl-2 and the proliferative index in either adenomas or carcinomas (*P*>0.05, *P*>0.1 in adenomas and carcinomas, respectively; data not shown). There was an inverse correlation between Bcl-2 and p53 in both adenomas and carcinomas. This trend was even more striking in carcinomas (*P*<0.005) than in adenomas (*P*<0.05; Table 4). Cell proliferation and apoptosis were positively correlated in both adenomas (*P*<0.02) and carcinomas (*P*<0.05; Table 4).

Discussion

The Ki-67 antigen is a nuclear protein that is expressed in proliferating cells during late G_1, S, M, and G_2 phases of the cell cycle, but is consistently absent in G_0 phase cells [17]. Like other methods, such as bromodeoxyuridine (BrdU) labelling, analysis of argyrophilic nuclear organiser region, proliferating cell nuclear antigen, and flow cytometry, it reflects cell proliferative activity. In the present study, the proliferative index increased significantly from mild to moderate, and from moderate to severe dysplasia. This is in agreement with reports by other workers, in which the BrdU labelling index was enhanced significantly from low- to high-grade dysplasia in human colorectal adenomas [10]. The cell apoptotic index also showed a progressive increase from mild to moderate and moderate to severe dysplasia. However, this trend did not reach statistical significance. Therefore, although cell proliferation and apoptosis show a linear correlation in the development of carcinoma, the programmed cell death rate does not catch up with that of cell proliferation, which may provide a cell kinetics rationale for the progression of adenomas from low- to high-grade dysplasia and the transition from adenoma to carcinoma. Expression of p53 protein and mutation of *p53* gene have been shown to be significantly correlated in colorectal cancer [18]. To minimise the possibility of registering overexpression of wild-type p53, only cases with 10% or more of tumour cells showing distinct nuclear staining were regarded as positive. In the present study, p53 expression was associated with proliferation but not with apoptosis in both adenomas and carcinomas, which is in agreement with other findings [19, 20]. This indicates that mutant *p53* may be more closely related to the modulation of cell proliferation than of apoptosis during the development of colorectal carcinoma.

Bcl-2 protein has been shown to inhibit apoptosis in a variety of experiments [21]. We demonstrated a positive correlation between Bcl-2 expression and lower apoptotic index in adenomas, but not in carcinomas. We previously reported that Bcl-2 expression in carcinomas was significantly lower than in adenomas [13]. These results suggest that the role of Bcl-2 in inhibiting apoptosis is more important in adenomas than in carcinomas. Moreover, Bcl-2 expression was inversely correlated with p53 expression in both adenomas and carcinomas. Similar results were also reported in another analysis of colorectal tumours [20] and in one of breast cancer [22]. Mutant p53 has been shown to down-regulate Bcl-2 expression in some cancer cell lines [5]. However, there is increased expression of Bcl-2 in p53-deficient mice [6]. Therefore,

mutant p53 may be directly or indirectly responsible for the down-regulation of Bcl-2 expression in colorectal carcinomas.

References

1. Eastwood GL (1995) A review of gastrointestinal epithelial renewal and its relevance to the development of adenocarcinomas of the gastrointestinal tract. J Clin Gastroenterol 21 [suppl]:S1–S11
2. Sachs L, Lotem J (1993) Control of programmed cell death in normal and leukemic cells: new implications for therapy. Blood 82:15–21
3. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J (1992) Induction of apoptosis by wild type p53 in human colon tumour derived cell line. Proc Natl Acad Sci USA 89:4495
4. Lotem J, Sachs L (1993) Regulation by Bcl-2, c-Myc and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation competent and defective myeloid leukemic cells. Cell Growth Differ 4:41–47
5. Haldar S, Negrini M, Monna M, Sabbioni S, Croce C (1994) Down-regulation Bcl-2 by p53 in breast cancer cells. Cancer Res 54:2095–2097
6. Miyashita T, Krajewshi S, Krojewshi M, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D (1994) Tumour suppressor p53 is a regulator of Bcl-2 and Bax expression in vitro and in vivo. Oncogene 9:1799–1805
7. Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D (1994) Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene 9:1791–1798
8. Wang Y, Szekely L, Okan I, Klein G, Wiman KG (1993) Wild-type p53-triggered apoptosis is inhibited by bcl-2 in a V-myc-induced T-cell lymphoma line. Oncogene 8:3427–3431
9. Sunter JP, Hull DL, Appleton DR, Watson AJ (1980) Cell proliferation of colonic neoplasms in dimethylhydrazine-treated rats. Br J Cancer 42:95–102
10. Risio M, Coverlizza S, Ferrari A, Candelaresi GL, Rossini FP (1988) Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas and adenocarcinomas of the large bowel. Gastroenterology 94:899–906
11. Stryker SJ, Wolff BG, Culp CE, Libbe SD, Ilsstrup DM, MacCarty RL (1987) Natural history of untreated colonic polyps. Gastroenterology 93:1009–1013
12. WHO (1989) Histological typing of intestinal tumours. In: Jass JR, Sobin LO (eds) International histological classification of tumours, 2nd edn, no 15. Springer, Berlin Heidelberg New York
13. Hao XP, Ilyas M, Talbot IC (1997) Expression of Bcl-2 and p53 in the colorectal adenoma-carcinoma sequence. Pathobiology 65:140–145
14. Du MQ, Singh N, Husseuin A, Isaacson PG, Pan LX (1996) Positive correlation between apoptotic and proliferative indices in gastrointestinal lymphomas of mucosa-associated lymphoid tissue (MALT). J Pathol 178:379–384
15. Keer JFR, Winterford CM, Harmon BV (1994) Apoptosis. Its significance in cancer and cancer therapy. Cancer 73:2013–2026
16. Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306
17. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by monoclonal antibody Ki-67. J Immunol 133:1710–1715
18. Costa A, Marasca R, Valentinis B, Savarino M, Faranda A, Silvestrini R, Forelli G (1995) p53 gene point mutations in relation to p53 nuclear protein accumulation in colorectal cancers. J Pathol (Lond) 176:45–53
19. Baretton GB, Diebold J, Christoforis G, Volgt M, Muller C, Dopfer K, Schneiderbanger K, Schmidt M, Lohrs U (1996) Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Cancer 77:255–264
20. Sinicrope FA, Ruan SB, Cleary KR, Stephens C, Lee JJ, Levin B (1995) bcl-2 and p53 oncoprotein expression during colorectal tumourigenesis. Cancer Res 55:237–241
21. Korsmeyer S (1992) bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 80:879–886
22. Silvestrini R, Veneroni R, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F, Veronesi U (1994) The bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. J Natl Cancer Inst 86:499–504