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Bcl-2 expression in colorectal tumours. Correlation with p53, mdm-2, Rb proteins and proliferation indices

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Summary. Immunostaining for bcl-2 protein was performed in 27 colorectal adenomas and 108 colorectal adenocarcinomas. The aim of the study was to determine bcl-2 expression in correlation with p53, mdm-2 and Rb expression, with proliferation indices (Ki-67-LI, PCNA-LI) as well as with conventional clinicopathological variables. A higher proportion of adenomas (30.8%) than carcinomas (16.7%) expressed bcl-2 and conversely, a lower proportion of adenomas (7.4%) than carcinomas expressed p53 (57.1%), the difference being statistically significant ($p<0.0001$). No correlation of bcl-2 expression with p53 expression (parallel or inverse) as well as with the other parameters studied was observed in any tumour. The bcl-2^+/p53^- subgroup of cancers showed a trend for correlation with negative lymph node status. Our data suggest, that bcl-2 expression may be involved in the early phase of colorectal carcinogenesis regardless of p53 status, while p53 function may be involved in a late stage of the adenoma-carcinoma sequence. P53 is apparently not involved in the regulation of apoptosis in the colorectal neoplasias or perhaps bcl-2 expression, as an early event in colorectal tumours, may occur before changes of p53 take place. Tumours with bcl-2^+/p53^- immunophenotype are frequently associated with negative lymph node status and seem to have a less aggressive behavior.

Key words: Bcl-2, p53, mdm-2, Rb, Colorectal tumours

Introduction

Several genetic aberrations have been implicated in tumorigenesis including activation of cellular protooncogenes or inactivation of tumour suppressor genes (Bishop, 1987). These alterations result in accelerated rates of cell division, decreased rates of cell death or both (McDonnell, 1993).

Bcl-2 is an oncogene involved in the regulation of cell death by inhibiting programmed cell death in physiological and neoplastic conditions (Reed, 1994). Its role, as a new kind of oncogene, has been primarily investigated in haematological malignancies and concretely in follicular B-cell lymphomas. In these lymphomas, overproduction of both bcl-2 mRNA and protein has been associated with a t(14;18) chromosomal translocation (Tsujimoto et al., 1985). Bcl-2 expression has also been investigated in non-lymphoid tissues, such as prostate (McDonell et al., 1992), lung (Ikegaki et al., 1994), breast (Silvestrini et al., 1994), thyroid (Pilotti et al., 1994), ovarian (Henriksen et al., 1995) and endometrial (Chhieng et al., 1996) tumours, as well as in gastrointestinal tumours (Flohil et al., 1996; Krajewska et al., 1996; Watson et al., 1996; Yao et al., 1999).

Other important genes that play a role in the regulation of apoptosis are the tumour suppressor genes p53 and Rb (Hooper, 1994); the latter has been suggested to induce apoptosis via p53. Wild-type p53 has been demonstrated to induce apoptosis (Ginsberg et al., 1991) and the bcl-2 gene is a transcriptional target for wild-type p53 which decreases bcl-2 protein levels both *in vitro* and *in vivo* (Miyashita et al., 1994). Furthermore, mutant p53 has been shown to inhibit bcl-2 expression in some cancer cell lines (Haldar et al., 1994). There is now a growing list of proteins which potentially involve p53 in pathway which regulate apoptosis. One of these proteins is the murine double-minute-2 (mdm-2) gene product that has been shown to couple wild-type and mutant p53 protein bringing about function inactivation of the p53 gene (Wu et al., 1993).

Recently, some studies in colorectal tumours have revealed evidence of the adenoma-carcinoma sequence with regard to bcl-2 and p53 overexpression. It has been reported that bcl-2 expression was reduced in carcinomas compared with adenomas (Flohil et al., 1996), suggesting a down-regulation of its expression when adenomas progress to carcinomas. In addition, bcl-2 expression has also been described as being associated with a better prognosis in cases of colorectal carcinomas (Ofner et al., 1995). Mutation and overexpression of p53 occurs late in colorectal carcinogenesis before the transition of an adenoma to a carcinoma and p53 protein accumulation has been associated with an aggressive behavior of colon carcinomas (Smith et al., 1996).

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Furthermore, p53 expression has been reported to be inversely correlated with bcl-2 expression in colorectal tumours (Watson et al., 1996; Hao et al., 1998).

The aim of this study was to examine bcl-2 expression in a series of colorectal adenomas and carcinomas, in an attempt to clarify the role of this gene in the development and progression of colorectal tumours. We also searched for a possible correlation between bcl-2 expression and p53, mdm-2, Rb expression as well as with proliferation indices (Ki-67, PCNA) and clinicopathological data (age, sex, tumour size, grade and Dukes' stage).

Materials and methods

A total of 27 colorectal adenomas and 108 colorectal adenocarcinomas were obtained either from surgical resection specimens or by endoscopic biopsy. Specimens were collected shortly after surgery and were snap frozen in isopentane liquid nitrogen using an embedding medium for frozen tissue specimens, O.C.T. compound (Tissue Tek-Miles). The frozen specimens were stored at -80 °C until processing. One parallel sample was fixed in 10% formalin for 24 hours and was subsequently processed with routine techniques and embedded in paraffin. Histological slides from paraffin-embedded tumour tissue were classified according to their pathological features following the World Health Organization (WHO) criteria (Jass and Sobin, 1989). Thus, according the histological growth type of adenomas, they were separated into three categories: tubular [n=14 (52%)], villous [n=4 (15%)] and tubulo-villous [n=9 (33%)]. With regard to the grade of dysplasia, there were 18 adenomas (67%) with low grade (mild, moderate) dysplasia and 9 adenomas (33%) with high grade (severe) dysplasia. Histologically, all colorectal carcinomas were adenocarcinomas. The age of the patients at time of surgery ranged from 26 to 86 years old. According to the WHO criteria, the tumour grade was high in 26 cases (24%), moderate in 73 cases (68%) and low in 9 cases (8%). In addition, according to Dukes' stage there were 11 cases of stage A (10%), 54 cases of stage B (50%) and 43 cases of stage C (40%).

Immunohistochemical analysis was performed on 5μm tissue sections by the Avidin-Biotin Complex immunoperoxidase (ABC) technique. In brief, sections were deparaffinized in xylene and dehydrated. To unmask the epitopes of bcl-2, we microwaved the sections in 10mM citrate buffer, pH 6.0, whereas to unmask the epitopes of p53, mdm-2 and Rb, we microwaved the sections in T buffer. PCNA unmasking was achieved without using the heat-mediated antigen retrieval method. Then, the sections were treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase. Sections were incubated for 20 min with normal serum and subsequently the sections were incubated overnight with the primary monoclonal antibodies, anti-bcl-2 (M0887, Dako, diluted 1:40), anti-p53 (DO-7, IgG2b, Dako, diluted 1:80), anti-mdm-2 (Ab-1, Oncogene, diluted 1:80) and anti-Rb (Ab-5, Oncogene, diluted 1:80), while the incubation with anti-PCNA (PC-10, Dako, diluted 1:50) was 1 hour. After a PBS washing, the preparations were incubated with biotinylated anti-mouse IgG. The three-step immunoperoxidase technique using ABC (Dako) was performed according to the procedure described by Hall et al. (1990). The staining colour was developed with diaminobenzidine. Thereafter, the sections were lightly counterstained with Mayer's haematoxylin and mounted.

For Ki-67 immunostaining, 5 μm-thick frozen cryostat sections were air-dried, fixed in absolute acetone and stained using a sensitive two-step indirect immunoperoxidase technique. The sections were incubated with monoclonal antibody against Ki-67 (Dako, diluted 1:10) for 30 min in a moist chamber. After washing in PBS, the frozen sections were incubated for 30 min with a rabbit anti-mouse and for another 30 min with a swine anti-rabbit peroxidase conjugate. A diaminobenzidine-H_2O_2 substrate was used to visualize the immunoreactivity.

Bcl-2 immunostaining was scored as the percentage of cells exhibiting definite cytoplasmic immunoreactivity in at least 2000 neoplastic cells encountered in 20 representative high-power fields. Immunoreaction of bcl-2 was defined as negative when few (<5%) of cells were positive, positive when a few to many (5-50%) of cells were positive and positive when many to all (>50%) of cells were positive. Immunoreactivity for p53 and Rb was scored as follows: no immunoreactivity or positive staining in less than 5% of tumour cells; positive staining in 5-50% of tumour cells with a mainly focal distribution; positive staining in more than 50% positive tumour cells. Because a few cases displayed nuclear positivity for mdm-2, every stained nucleus was considered positive, irrespectively of intensity, and therefore, tumours were classified as mdm-2 negative and mdm-2 positive. The evaluation of Ki-67 and PCNA immunostaining was calculated as the percentage of positive tumour cells in relation to the total number (about 1000) of tumour cells. The percentage of positively-stained cells was recorded as Ki-67-LI (labelling index) and PCNA-LI and for statistical analysis the cases were divided into two groups (<50% and >50% of cells positive) according to the estimated number of positive cells. The expression of Ki-67 was evaluated only in 68 from the 108 cases of adenocarcinomas.

All tumours were independently scored by two observers. Statistical evaluation was confirmed using non parameter test for two or several independent samples or Spearman bivariable correlation. Multivariate analysis was carried out by the Cox model. A p value below 0.05 was regarded as statistically significant.

Results

Bcl-2 immunostaining was typically cytoplasmic (Fig. 1). In "normal" colonic epithelium adjacent to adenomas and adenocarcinomas, bcl-2 expression was observed at the base of crypts in most examined samples. In adenomas, positive bcl-2 staining of dysplastic epithelial cells was observed in 30.8% of the cases examined. Of these positive adenomas, 3 were classified as villous, 3 as tubular and 2 as tubulovillous; 4 cases contained areas with low grade dysplasia (1 case with mild and 3 cases with moderate), and 4 cases with high grade dysplasia. No relation was found between bcl-2 immunoreactivity and histological growth type or grade of dysplasia. In comparison with adenomas, the rate of aberrant immunohistochemical bcl-2 expression in adenocarcinomas was lower (16.7%) but this difference was not statistically significant. In addition, no relationship was found between bcl-2 expression and the patient's age, sex, tumour size, grade or Dukes' stage (Table 1).

P53 protein overexpression was not demonstrated in "normal" colorectal mucosa, but there were 2 cases of adenomas (7.4%), one villous and one tubular, with

Table 1. Bcl-2 expression and clinicopathological data in colorectal carcinomas.

	Bcl-2 expression (%)			p value
	<5	5-50	>50	
Age				
<60	28	7		NS
>60	58	10	5	
Sex				
female	42	8	4	NS
male	45	6	3	
Tumour size				
<5	38	7	7	NS
>5	45	6	5	
Grade				
G1	21	3	2	NS
G2	59	11	3	
G3	9			
Dukes' stage				
A	8	1	2	NS
B	47	5	2	
C	35	8		

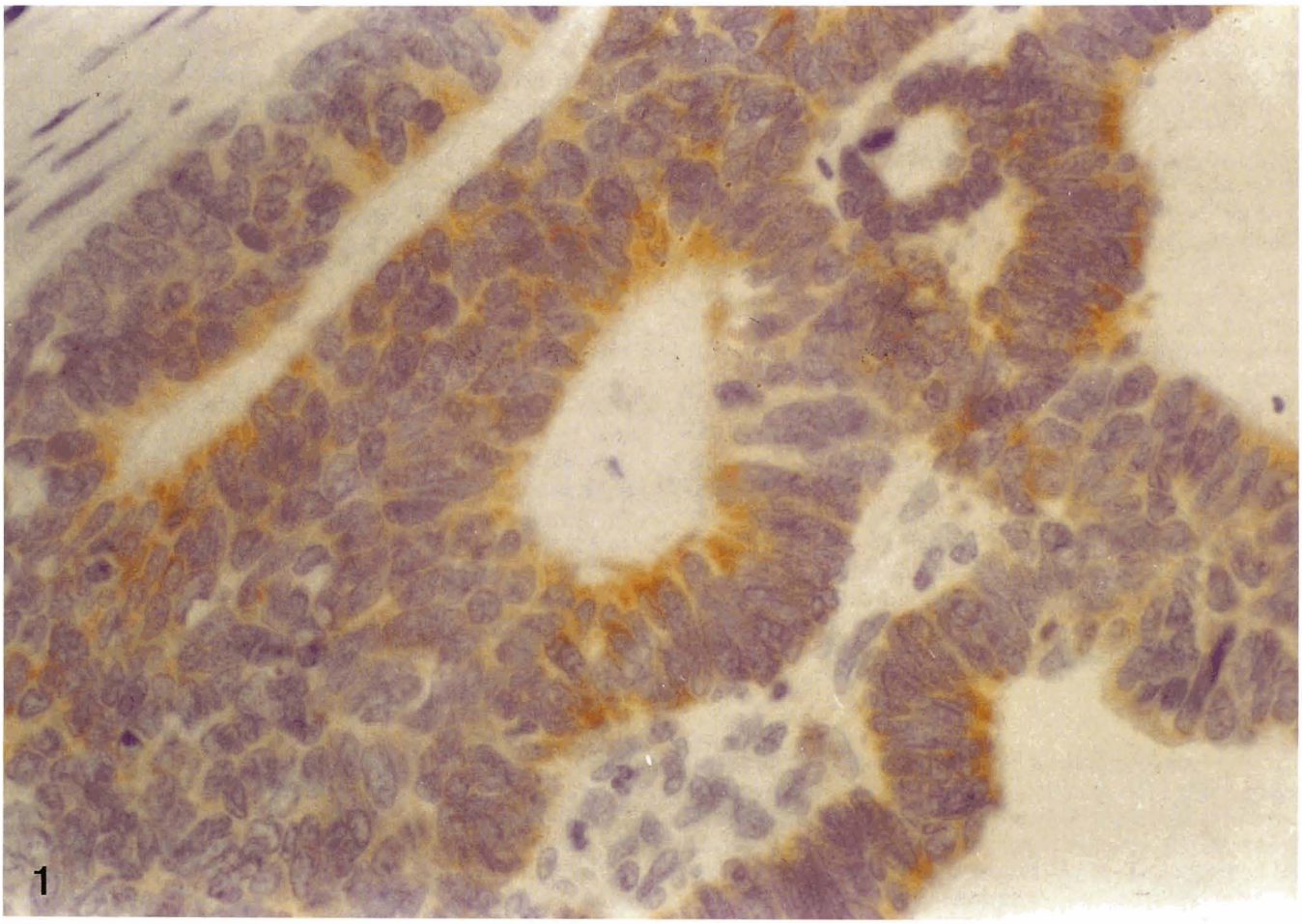

Fig. 1. Bcl-2 expression in a well differentiated adenocarcinoma, Dukes' stage B. ABC, x 200

Table 2. Bcl-2 expression and potential prognostic variables in colorectal carcinomas.

	Bcl-2 expression (%)			p value
	< 5	5-50	>50	
p53 (%)				
<5	36	5	2	
5-50	14	3	2	NS
>50	37	7	2	
mdm-2				
(-)	81	13	3	NS
(+)	7	3	1	
Rb (%)				
<5	42	5	2	NS
5-50	36	5	2	
>50	12	4		
Ki-67(%)				
<50	28	4	3	NS
>50	25	3	5	
PCNA(%)				
<50	15	3	3	
>50	71	13	3	

areas of moderate and severe dysplasia and 65 cases of adenocarcinomas (57.1%) having the evidence of p53 nuclear overexpression. This difference of p53 immunostaining between adenomas and carcinomas was statistically significant ($p<0.0001$), but there was no statistical correlation between bcl-2 expression and p53 immunoreactivity in any of these tumours (Table 2). Conversely, the bcl-2^+/$p53^-$ subgroup showed a trend for correlation with negative lymph node status. Mdm-2 expression was detected in 10.8% of adenocarcinomas investigated and only two cases of adenomas had a small number of mdm-2-positive cells (<10%). Both of these cases were tubular and of low grade dysplasia. Rb protein was expressed in 54.5% of adenocarcinomas and in 76.9% of adenomas. Of these positive adenomas 7 were villous, 8 tubular and 5 tubulovillous of low and moderate dysplasia. Neither mdm-2 nor Rb protein expression was correlated with bcl-2 expression. The mean value of Ki-67 and PCNA index was higher in carcinomas (45.54±16 and 67.54±21.74 respectively) than in adenomas (29.8±13 and 30.17±25.34 respectively). No statistically significant correlation between the proliferative activity and bcl-2 expression was observed in either adenomas or carcinomas.

Discussion

Sequential studies of transformation in a variety of colorectal lesions (ranging from reactive to neoplastic or from adenoma to carcinoma) indicate that carcinogenesis is a multistep process involving mutation. This includes the activation of oncogenes inducing growth promotion, as well as inactivation of growth suppressor genes. The final consequence of this genetic dysregulation is the loss of control of cell proliferation and differentiation. During the past decade, apoptosis has attracted more interest than any other biomedical field. The reason for this research focus on apoptosis is not only because it represents an important biological principle in tissue development and homeostasis, but also that its regulation appears to be perturbed in several major diseases, including cancer (Reed, 1997). One of the most important developments in apoptosis research, has been the identification of the roles played by a variety of oncogenes and suppressor genes (bcl-2, c-myc, ras, p53, Rb). In this study we investigated the expression of bcl-2 protein in a series of benign and malignant epithelial colorectal tumours. Moreover, we studied the correlation of bcl-2 with p53 accumulation, Rb and mdm-2 protein expression as well as with proliferative activity and conventional clinicopathological parameters. Though many details are missing, we are beginning to understand the biological mechanism of bcl-2 action (Wang and Reed, 1998). Several studies have suggested the possible role of bcl-2 for the regulation of cellular events in normal tissue and as a possible initial step toward malignancy in multistep carcinogenesis. In colonic tissue, the physiological expression of bcl-2 protein is confined especially to the stem cells and at the base of crypts (Hockenbery et al., 1991; Hague et al., 1994). Evidently, the role of bcl-2 is to protect the stem cells and for the renewal and repair abilities of the epithelium from apoptosis (Hockenbery et al., 1991). There are studies which suggest that the majority of colorectal cancers express bcl-2 (Hague et al., 1994), while in other studies this expression is observed in a lower proportion (Ofner et al., 1995). Sinicrope et al. (1995) reported the first data concerning the importance of bcl-2 in colorectal tumorigenesis: in 71% of adenomas and in 67% of adenocarcinomas, bcl-2 immunoreactivity was detected. Similar results have been reported in other studies although the reduction of bcl-2 expression in carcinomas compared with adenomas was more apparent (Hao et al., 1997). There are also data which suggest, that bcl-2 expression and a low rate of apoptosis were seen more often in villous than tubular adenomas. These studies suggest, that bcl-2 is involved in the prevention of apoptosis in large bowel epithelium and that, if overexpression is important in the carcinogenetic process, it probably occurs as an early event.

In our study, we found that a lower proportion of adenomas and carcinomas expressed bcl-2 protein: in 30.8% of adenomas and in 16.7% of cancers bcl-2 immunostaining was observed. In comparison to other studies, we had discrepancies in our results. Perhaps these discrepancies may be related to differences in methodological procedures. However, in all studied cases, bcl-2 immunoreactivity in lymphocytes was used as an internal positive control. In good accordance with the observations published recently (Watson et al., 1996), no relation was found between bcl-2 immunoreactivity and histological growth type and grade of atypia/dysplasia of adenomas. In addition, in the group of adenocarcinomas no relationship was

observed between bcl-2 immunostaining and patient's age or sex, tumour size, grade and stage (Table 1).

The nuclear accumulation of p53 protein has been reported in many kinds of tumours, including colorectal cancer, in relation to p53 gene mutations (Costa et al., 1995). In the colon, p53 mutations are detected frequently in invasive carcinomas and rarely in adenomatous polyp, (Ichii et al., 1993; Houlston and Tomlinson, 1997). In a recent study, concerning analysis of genetic alterations in adenomas of familiar adenomatosis polyposis, p53 mutations have been reported in only two of 76 adenomas with moderate and severe dysplasia (Ichii et al., 1993). These results suggest, that inactivation of the p53 gene are associated with later events, such as malignant transformation or progression to carcinomas. Recent work indicates that an important aspect of p53 function involves the induction of apoptosis, and it now appears clear that mutations in p53 promote tumorigenesis by interfering with programmed cell death. There are reports which argue that p53 may induce apoptosis, at least in part, by regulating transcription of bcl-2 family members. Under certain conditions, p53 induction leads to up-regulated expression of the apoptosis-promoting protein Bax, whilst a death-repressing member of the same protein family, bcl-2, is down-regulated (Miyashita et al., 1994): indeed a p53-dependent negative response element has been identified in the bcl-2 gene.

P53 expression has been reported to be inversely correlated with bcl-2 expression in colorectal adenomas and carcinomas (Watson et al., 1996; Hao et al., 1998), while another study has suggested that this correlation is confined only in adenomas (Sinicrope et al., 1995). In the current study, to minimize the possibility of registering overexpression of wild-type p53, only cases with 5% or more of tumour cells showing distinctive nuclear staining were regarding as positive. Overexpression of p53 protein was found in 7.4% of adenomas and in 57.1% of adenocarcinomas studied. In comparison with adenomas, the rate of aberrant immunohistochemical p53 expression in carcinomas was statistically significantly higher. In accordance with results from other studies in colorectal tumours (Baretton et al., 1996; Yao et al., 1999), we found no statistical correlation between bcl-2 and p53 accumulation in both adenomas and carcinomas. These observations probably mean that p53 is apparently not involved in the regulation of apoptosis in the colorectal neoplasias or perhaps, that bcl-2 expression, as an early event in colorectal tumours, may occur before changes of p53 take place. On the other hand, in the series of carcinomas there was a trend for correlation between the bcl-2^+/$p53^-$ subgroup with negative lymph node status. It is possible that tumours with bcl-2^+/$p53^-$ phenotype imply a less aggressive pathway of neoplastic transformation.

Nuclear mdm-2 protein immunoreactivity was observed in 10.8% of adenocarcinomas while only two cases of adenomas had a small number of positive neoplastic cells. Heterogenous nuclear staining for Rb protein was detected in 54.5% of adenomas and in 76.9% of adenocarcinomas. Neither protein was correlated with bcl-2 expression of any tumours. Perhaps, these proteins contribute in different ways to the regulation of apoptosis.

The proliferative activity, as measured by Ki-67 and PCNA immunoreactivity, increased significantly on progression from adenoma to carcinoma, but did not vary significanly within the separate adenoma and carcinoma groups. It has been reported that in the series of colorectal carcinomas the grade of Ki-67 index was correlated in a statistically significant manner with the rate of apoptotic cells (Baretton et al., 1996). These observations support the idea that increased proliferation is often accompanied by increased apoptosis. In our study, although a trend toward an inverse correlation between bcl-2 with Ki-76 and PCNA index was found, no statistically significant correlation between these parameters was observed in either adenomas or carcinomas.

In conclusion, bcl-2 protein is expressed in colorectal adenomas and carcinomas with a higher incidence in adenomas. This fact probably means, that bcl-2 expression is characteristic of the early stage of colorectal tumorigenesis. Since bcl-2 expression did not correlate with p53 status, it can be concluded that in these tumors the regulation of apoptosis is quite complex and cannot simply be reduced to the assessment of bcl-2 gene expression or p53 accumulation. However, in bcl-2^+/$p53^-$ cancers , the strong but not statistically significant correlation between this phenotype and negative lymph node status shows, that there is a subgroup of colorectal adenocarcinomas which may have a better biological behavior. Further studies, perhaps of other regulators of apoptosis, are required before the value of bcl-2, as prognostic indicator, is established. In addition, as more anti-apoptotic mechanisms are uncovered, new potential therapeutic targets will be defined which should enable a more effective and more specific treatment of colorectal cancer. The elucidation of these mechanisms is an interesting issue for future investigation.

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