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ARTICLE

Correlation of bcl-2 Oncoprotein Immunohistochemical Expression with Proliferation Index and Histopathologic Parameters in Colorectal Neoplasia

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The *bcl-2* oncogene plays an important role in carcinogenesis by inhibiting cell death (apoptosis). It was initially discovered in follicular B cell lymphoma with t(14,18), and subsequently found in other malignant and premalignant lesions. Alteration of the normal controls of cell proliferation is also a significant factor in the multistep process of tumorigenesis. The proliferative activity of a given lesion is commonly evaluated by MIB1, a monoclonal antibody to Ki67 proliferation antigen. Immunohistochemical (IHC) staining expression of bcl-2 and Ki67 was retrospectively investigated in a series of 52 colorectal carcinomas and 56 adenomas according to the avidin-biotin-complex method. The aim of the study was twofold: 1) to investigate any correlation between MIB1 and bcl-2 immunostaining expression in colonic adenomas and carcinomas, 2) to identify any relationship between either marker and several histopathologic parameters including tumor size, pathologic stage, lymph node metastasis, angiolymphatic invasion, tumor grade and differentiation in colon carcinomas. Bcl-2 was consistently higher in adenomas than in carcinomas. There were 44/56 (78.6%) adenomas, and 27/52 (51.9%) carcinomas positive for bcl-2 (p=0.004). The mean Ki67 labeling index (LI) was 30.05±7.6 and 38.12±11.01 in adenomas and carcinomas, respectively (p=0.0001). Expression of bcl-2 in carcinoma was significantly associated with a lower mean Ki67 LI and with favorable histopathologic parameters. We conclude that bcl-2 oncoprotein expression is probably an early step in the process of colon carcinogenesis, and its expression may be associated with a favorable clinical course. Furthermore, an inverse relationship exists between bcl-2 and Ki67 in colonic neoplasia. Evaluation of bcl-2 and Ki67 IHC expression in colonic carcinoma should be performed prospectively to determine if their expression is of value in predicting the clinical course in these patients. (Pathology Oncology Research Vol 5, No 4, 273–279, 1999)

Keywords: colon carcinoma, adenoma, bcl-2, Ki-67, proliferation index, immunohistochemistry

Introduction

Regulation and maintenance of balance between cell death and cell proliferation are very critical for normal and neoplastic tissue homeostasis. The bcl-2 oncoprotein functions as a programmed cell death (apoptosis) inhibitor, and therefore, prolongs the cell life span. It thus increases the risk of acquiring other unfavorable changes such as chromosomal abnormalities, DNA-damaging agents and viral infection rendering the cells more susceptible to malignant transformation.[1-10]

The *bcl-2* gene is located on chromosome 18q21. The bcl-2 oncoprotein was initially discovered, and is most commonly associated, with B cell follicular lymphoma with t(14,18)[(4)]. Subsequently, it was found in other lymphoproliferative diseases without t(14,18), in normal epithelia with active proliferative compartment, and in various epithelial

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neoplasias.[4-7,9] Furthermore, its association with precancerous lesions suggests a role in the early stage of carcinogenesis.[1,5,7] Currently, it is recognized that *bcl-2* is a member of a group of genes with sequence homology known as the *bcl-2* gene family which regulates cell death and survival. A related gene, *bax*, opposes bcl-2 action and hence accelerates cell death. The relative levels of these two genes seems to regulate cell survival.

Tumorigenesis is a multistep process which begins with abrogation of normal controls of cell proliferation; thus, measurement of the tumor growth fraction may be clinically important.[11] MIB1 stains the Ki67 proliferative nuclear antigen and studies have shown that MIB1 proliferative index value may provide prognostic information.[12-22.]

In the current study, we evaluated the Ki67 LI and bcl-2 oncoprotein IHC expression in colonic adenomas and carcinomas to explore their role in the carcinogenesis process, and to identify any possible relationship between Ki67 and bcl-2 IHC expression. Correlation of Ki67 and bcl-2 IHC expression with histopathologic parameters was also investigated.

Material and Methods

Surgical specimens of colorectal carcinomas (52) and adenomas (56) diagnosed during the period of January-June 1997 were retrieved from the archives of the Pathology Department at Grace Hospital, Detroit, Michigan. Sections of paraffin blocks of the most representative areas were made for immunohistochemical staining. Histologic grading of the carcinomas as well, moderate and poorly differentiated followed the WHO classification criteria. The pathologic stage was determined according to Duke's staging system. The carcinomas were carefully examined for angiolymphatic invasion and number of lymph node metastases on the H&E sections. The size of the tumor was obtained from the gross pathologic record. The adenomas were divided into groups based on size (≤ 1cm, > 1cm), histologic type (tubular, tubulovillous and villous), and absence or presence of high grade dysplasia.

The IHC staining procedure for bcl-2 and MIB1 monoclonal antibodies followed the avidin-biotin-peroxidase complex method after microwave heating to maximize antigen retrieval. Four μ*m* thick sections from the formalin-fixed, paraffin-embedded tissues were made.

For bcl-2 (Dako Corporation, Carpinteria, CA), tissue sections were deparaffinized then microwaved on high in citrate buffer for 15 min and left for an additional 15 min in hot buffer. They were rinsed in water and placed in horse serum for 10 min. Sections were then incubated with primary antibody diluted 1:40 for 2 h, biotinylated anti-mouse (Vector Laboratories, Burlengcome, CA) for 10 min. After color development with 3-amino-9-ethylcarbazole, sections were counterstained with hematoxylin. Negative controls consisted of equivalently diluted nonimmune mouse IgM. Section of a human lymph node served as a positive control for bcl-2 protein.

For MIB1 (AMAC Inc., Westbrook, ME), the sections, after deparaffinization and blocking of endogenous peroxidase, were placed in 10 mM, pH 6.0, citrate buffer and processed in a microwave on high for 15 min. They were allowed to stand in hot buffer for an additional 15 min. They were then incubated with primary antibody, MIB1 diluted 1:20 for two hr, biotinylated anti-mouse (Vector Laboratories) diluted 1:200 for 10 min and avidin-biotin-complex peroxidase reagent for 10 min. After color development with 3-amino-9-ethylcarbazole, the sections were counterstained with hematoxylin. Section of colonic adenocarcinoma known to be positive for MIB1 served as a positive control.

The bcl-2 and MIB1 stained slides were evaluated on light microscopy without knowledge of the examining pathologist of the clinical outcome or histopathologic findings. With regards to bcl-2, the tumor cells were considered positive when they displayed a distinct cytoplasmic reaction. Bcl-2 staining was semiquantitatively divided into: low, moderate and high when < 25%, 25–50% and > 50% of the neoplastic cells were immunoreactive, respectively. As for the MIB1 staining, the number of tumor cells with distinct nuclear staining was recorded after counting 500 tumor cells in consecutive high power fields in the most reactive areas on the slide. Cells with questionable nuclear staining were discounted. The percentage of positive tumor cells was then calculated as MIB1 LI. Necrotic or thick areas and severely overlapping tumor cells were avoided during evaluation.

Statistical Methods – Chi-square tests were used to determine if clinical pathologic parameters (*e.g.*, size, differentiation, pathologic stage, lymph node metastasis, angiolymphatic invasion, dysplasia, *etc.)* correlated significantly with bcl-2 expression in carcinomas and adenomas. Fisher's exact test was used to determine statistical significance when small numbers were encountered in the contingency tables (*i.e.*, expected value of any cell less than 5). Comparison of mean Ki67 LI between groups was formally tested using t-tests (for two groups) and ANOVA (for more than two groups). When the overall F-test in ANOVA was significant, multiple comparisons were performed using Tukey's method.

Results

The patient population consisted of 55 men and 53 women ranging in age from 46 to 78 years. The 56 patients with adenomas ranged in age from 46 to 60 years (mean 51). The 52 patients with carcinoma ranged in age from 54 to 78 years (mean 62). The carcinomas were grouped according to size, differentiation, pathologic

Table 1. **Immunohistochemical expression of bcl-2 and Ki67 (MIB1) antibodies in colorectal carcinomas**

		No.	*Ki67 LI (mean ± S.D.)*	*p value*	*bcl-2 positive (%)*	*p value*
Size	≤ 2cm	14	32.5 ± 7.3	0.02	9 (64.2)	0.28
	> 2cm	38	40.2 ± 11.5		18 (47.3)	
Differentiation	well / moderate	42	35.7 ± 9.5	0.0007	20 (47.6)	0.29
	poor	10	48.3 ± 11.7		7 (70)	
Pathologic stage	Duke A	11	34.7 ± 7.8	0.17	7 (63.6)	0.10
	Duke B	28	37.3 ± 10.9		16 (57.1)	
	Duke C	13	42.8 ± 12.8		4 (30.7)	
Lymph node metastasis	0	26	35.3 ± 10.3	0.02	15 (57.7)	0.34
	≤ 3	16	37.5 ± 9.2		8 (50)	
	> 3	10	46.3 ± 12.4		4 (40)	
Angiolymphatic invasion	yes	11	37.5 ± 9.7	0.83	6 (54.5)	0.85
	no	41	38.3 ± 11.5		21 (51.2)	

stage, presence or absence of lymph node metastasis and angiolymphatic invasion (*Table 1*). In our study, all the carcinoma cases were of the usual gland-forming adenocarcinoma. There were 14 carcinomas ≤2 cm and 38 > 2cm. Well and moderately-well differentiated carcinomas were grouped together for simplicity and accounted for 42 cases, while poorly differentiated cases numbered 10. According to Duke's staging system, 11 carcinomas were stage A, 28 stage B and 13 stage C. Twenty-six cases had no lymph node metastasis, 16 cases had ≤3, and 10 cases had > 3 lymph node metastases. Angiolymphatic invasion (ALI) was present in 11 cases and absent in 41 cases.

There were 42 adenomas ≤1 cm and 14 were >1cm in size. With respect to the histologic type, there were 37 tubular, 13 tubulovillous and 6 villous adenomas. Eleven adenomas contained areas of high grade (HG) glandular dysplasia and the remaining 45 were of the usual low grade (LG) histologic type (*Table 2*).

Ki67 immunoreactivity

Ki67 (MIB1) immunostaining produced discernible diffuse or granular, brown nuclear staining, more accentuated in the nucleoli, with relatively uniform intensity. The positive epithelial cells were easily identified especially the mitotic cells. Also, intense nuclear staining was observed in the lymphoid cells and they served as internal control (*Figure 1*). Mean Ki67 LI in carcinoma was 38.12±11.01 compared to 30.05±7.6 in adenoma (p=0.0001) (*Table 3*). The mean Ki67 LI (±S.D.) was lower for carcinomas ≤2 cm than in those > 2 cm; 32.5% vs. 40.2%, respectively, and this was statistically significant (p=0.02). The mean Ki67 LI appeared to increase with decreasing degree of differentiation of carcinoma: 35.7±9.5 in well/moderately-well differentiated vs. 48.3±11.7 in poorly differentiated carcinomas (p=0.0007). With respect to the pathologic stage, mean Ki67 LI increased with advancing tumor stage: 34.7% in Duke's A vs. 42.7% in Duke's C (p=0.17).

Table 2. **Ki67 (MIB1) Labeling Index and bcl-2 immunohistochemical expression in colorectal adenomas**

		No.	*Ki67 LI (mean ± S.D.)*	*p value*	*bcl-2 positive (%)*	*p value*
Size	≤ 1cm	42	26.7 ± 5.1	< 0.0001	34 (80.9)	0.47
	> 1cm	14	39.4 ± 5.7		10 (71.4)	
Type	tubular	37	27.5 ± 5.4	0.0005	30 (81)	0.46
	tubulovillous	13	33.7 ± 9.7		10 (76.9)	
	villous	6	38.0 ± 5.8		4 (66.6)	
Dysplasia	low grade	45	27.9 ± 6.1	< 0.0001	36 (80)	0.69
	high grade	11	38.7 ± 6.8		8 (72.7)	

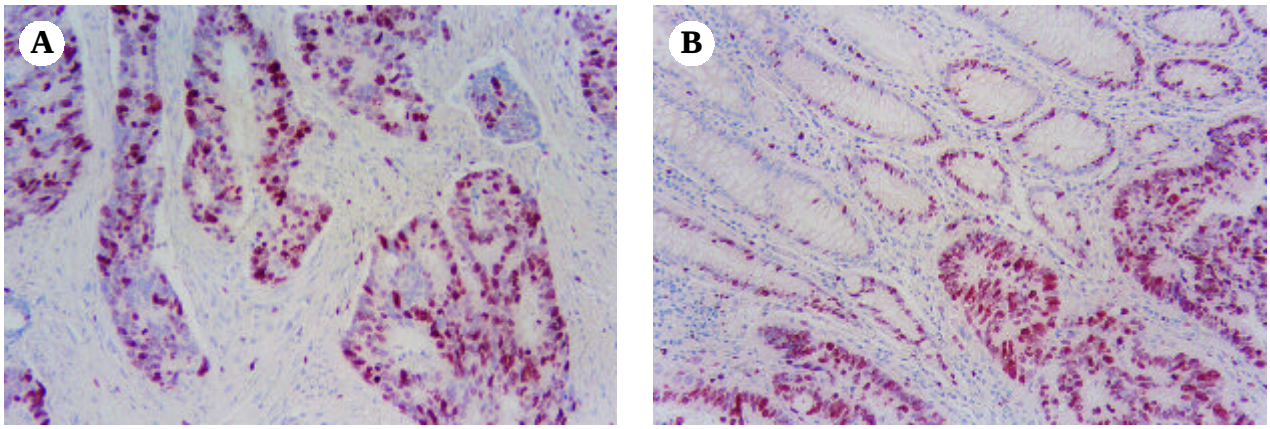

Figure 1. Intense nuclear staining is observed in a case of colon carcinoma to Ki67 (MIB1) immunostain (A) (x400) . Note that the reactivity is more intnese in the carcinoma areas compared to the adjacent normal mucosa where cells are positive mainly in the crypt base (B) (x100)

Interestingly, the number of lymph node metastases had a significant impact on the mean Ki67 LI values. Carcinomas with no lymph node metastasis had a 35.3±10.3 mean Ki67 LI, compared to 37.5±9.2 and 46.3±12.4 in those with ≤3 and >3 lymph node metastases, respectively. Comparing the results of cases with no lymph node metastasis to those with >3 lymph node metastasises, the difference was statistically significant (P=0.02). On the other hand, angiolymphatic invasion had no impact on mean Ki67 LI of the studied colon carcinomas: 37.5±9.7 with ALI and 38.3±11.5 in those lacking ALI (P=0.8).

When evaluating the results of Ki67 immunostaining in adenomas *(Figure 2.) (table 2)*, there were statistically significant differences. For example, adenomas >1 cm in size had a higher mean Ki67 LI (39.4±5.7) than adenomas ≤1cm (26.9±5.1) (P=0.0001). Also, adenomas with HG dysplasia had a higher mean Ki67 LI (38.7±6.8) than LG adenomas (27.9±6.1) (P=0.0001). With respect to the histologic type of the adenomas, the mean Ki67 LI correlated well with increasing amount of villous component: 27.5±5.4 in tubular, 33.7±9.7 in tubulovillous and 38.0±5.8 in villous (P=0.0005).

Bcl-2 immunoreactivity

The carcinomas had lower rate of bcl-2 positivity than the adenomas (51.9% vs. 78.6%), (P=0.004) (*Table 3*). Focal faint cytoplasmic staining was discounted. Lymphocytes in the stroma/lamina propria were consistently positive and served as an internal control. The staining pattern was not uniform in adenomas: strongly reactive areas alternated with mildly reactive areas. The intensity of cytoplasmic staining, also, varied from case to case. The nonadenomatous mucosa adjacent to the adenoma was largely negative except for the basal, regenerative glandular compartment. Similarly, carcinomas showed heterogenous staining without specific spatial pattern.

Most of the positive adenomas, 32 of 44 (72.7%), showed high (>50%) staining, while 7 (15.9%) had moderate staining (25–50%) and the remaining 5 (11.3%) had low staining (<25%). In contrast, most of the positive carcinomas, 15/27, (55.5%) displayed low immunoreactivity for bcl-2, while 7 (25.9%) were moderate and 5 (18.5%) showed high positivity.

Positive bcl-2 staining was more frequent in carcinomas ≤2 cm than those >2 cm: 9/14(64.2%) vs. 18/38 (47.3%), respectively, but this was not statistically significant (P=0.28) (*Table 1*). Also, bcl-2 was more frequently positive in well/moderately differentiated than in poorly differentiated carcinoma: 70% vs. 47.6%, respectively, (P=0.29). The results also showed that bcl-2 tended to be more often positive in early than in advanced stages of carcinoma: 63.6% in Duke's A vs. 30.7% in Duke's C (P=0.10). Bcl-2 was positive in 15/26 (57.7%) carcinomas without lymph node metastases as compared to 50% and 40% of those with ≤3 and >3 lymph node metastases, respectively. The results

Table 3. **Immunohistochemical expression of bcl-2 and Ki67 (MIB1) in colorectal adenomas and carcinomas.**

	No.	*Age (mean)*	*Ki67 LI (mean ± S.D.)*	*p value*	*bcl2 positive (%)*	*p value*
Adenocarcinoma	52	62	38.12 ± 11.01	0.0001	27 (51.9)	0.004
Adenoma	56	51	30.05 ± 7.6		44 (78.6)	

were not statistically significant when cases with no lymph node metastasis and those with > 3 node metastasises were compared (P=0.34). There were no apparent differences in bcl-2 staining in relation to the ALI, 6/11 (54.5%) carcinomas with ALI, and 21/41 (51.2%) of carcinomas without ALI were positive (P=0.85). Bcl-2 was positive in 30/37 (81%) tubular, 10/13 (76.9%) tubulovillous, and 4/6 (66.6%) villous adenomas, showing a trend of more frequent positivity in tubular than villous adenomas (p=0.46) (*Table 3*). Bcl-2 was more frequently positive in adenomas ≤1 cm (34/42, 80.9%) than in those > 1 cm those (10/14, 71.4%), but this was not statistically significant (P=0.47). With respect to the degree of dysplasia in the adenoma, bcl-2 was more frequently positive in LG adenomas (36/45, 80%) than in HG adenomas (8/11, 72%), but this was not statistically significant (P=0.69).

Discussion

Colorectal cancer is the second leading cause of cancer mortality in the United States. Quantification of cell proliferative activity in neoplasia is currently the subject of considerable investigation. The Ki67 is a nuclear antigen expressed in highest concentrations in all stages of the cell cycle but not in resting cells. MIB-1 is a monoclonal antibody that recognizes a fixation-resistant epitope of the Ki67 antigen and is, presently, widely used to estimate the proliferative fraction of neoplasias.[12,13,16,18,20]

Ki67 IHC staining pattern has been found to correlate well with tumor growth fraction and S phase fraction in various human malignancies, however, correlation with clinicopathologic parameters was inconsistent.[24-27] Some studies have shown significant correlation of Ki67 LI with several clinically important prognostic pathologic parameters in colorectal carcinomas such as tumor differentiation, metastatic disease and local invasiveness,[16,19,20] while other studies showed no such correlation.[20-22] Investigators have suggested that this lack of correlation is due to the considerable heterogeneity of colon carcinoma.[12,13,18]

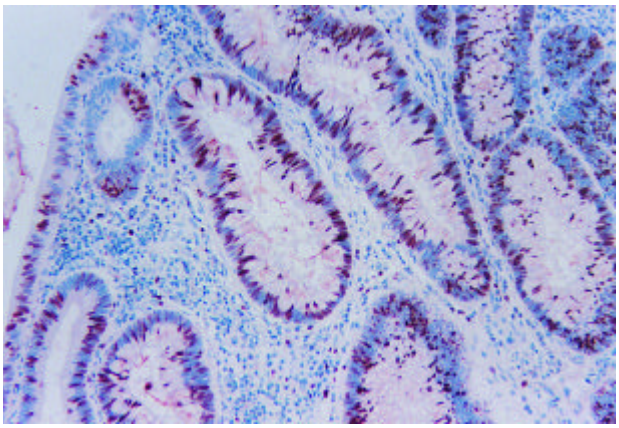

Figure 2. A tubular adenoma with readily visible positive nuclear staining to MIB1 in the neoplastic cells. (x400).

Our study demonstrated that mean Ki67 LI is significantly associated with poor prognostic histologic parameters of colorectal carcinoma. Higher Ki67 LI values were more frequently associated with lymph node metastases, poorly differentiated tumors, and tumors more than 2 cm in size (*Table 2*). This was statistically significant with p value of 0.02, 0.0007 and 0.02 respectively. Our study also demonstrated a difference in mean Ki67 LI with regards to Duke's pathologic stage. Although not statistically significant (p=0.17), it clearly showed a trend of increasing mean Ki67 LI with advancing stage of disease. ALI was the least correlating parameter with mean Ki67 LI. Mean Ki67 LI was 37.5 ± 9.7 in cases with ALI, and 38.3 ± 11.5 in those without ALI (P=0.83).

The results of our study are in agreement in some aspects with those of other investigators. For example, our findings are in concordance with Suzuki[16] who found increasing mean Ki67 LI with advancing Duke's stage in colon carcinoma. Our results are also in agreement with those of Kyzer and Gordon[20] (mean Ki67 LI positively correlates with metastatic disease status), and with those of Lanza[19] (mean Ki67 LI inversely correlates with the degree of tumor differentiation).

Previous studies showed a higher mean proliferative activity in colonic carcinoma than in adenoma.[11,21,22,28] Johnston et al[21] reported a mean Ki67 score of 45.5 for adenoma and 66.3 for carcinoma (p=0.001). In our study, the mean Ki67 LI was significantly higher in carcinoma (38.12±11.01) than in adenoma (30.05±7.6) (p=0.0001). The superficial areas of carcinomas, adenomas with HG dysplasia, and cells located near the gland lumina in these lesions were more frequently positive than those of the crypt base or lower compartment. However, in LG adenomas and normal mucosa, cells in the lower third were more consistently positive. We concur with previous investigators that Ki67 immunostaining can be used to characterize the various proliferative zones of normal colonic mucosa, adenoma and carcinoma.

Traditionally, increased cell proliferation was emphasized as the main biologic impetus leading to neoplasia, but recently, there has been a growing interest in the alteration of programmed cell death (apoptosis) as a possible pathway leading to malignancy. Apoptosis is a protective mechanism by removing senescent, DNA-damaged or diseased cells. Furthermore, it is currently believed that accumulation of multiple genetic defects that alter normal growth control, differentiation and programed cell death contribute to the development of colorectal carcinomas.[8] Direct evidence of the role of the latter is the bcl-2 gene which was initially observed in follicular B-cell lymphoma with t(14;18). Also, it has been recently demonstrated that both bcl-2 and p53 genes play key roles in the development

of colorectal carcinoma through interactive mechanisms of regulating apoptosis.[29]

The bcl-2 is a gene family involved in the regulation of cell death and survival by inhibiting apoptosis in physiologic and neoplastic conditions.[9] Studies have showed that bcl-2 is expressed in various neoplasias including lymphomas, breast, gastrointestinal, lung, prostate and uterine carcinomas and sarcomas.[2-6,9] It is thought that bcl-2 plays a role in colorectal carcinoma by blocking apoptosis in the early stage of the multistep carcinogenesis, thus keeping the cells alive and lending them vulnerable for further accumulation of gene abnormalities.[7] Moreover, bcl-2 expression was also seen in various precancerous and cancerous lesions, including colorectal, suggesting that its alteration occurs early in the sequence of molecular events leading to carcinoma.[6,8,9]

It has been recently reported that prognostic significance of bcl-2 expression is variable in solid tumors with favorable prognosis in breast and lung carcinomas and unfavorable in prostate carcinoma.[9] Barreton et al demonstrated that bcl-2 expression in colorectal carcinomas was associated with a better clinical course especially if p53 expression was absent[5] suggesting that neoplastic transformations related to inhibition of apoptosis may result in less aggressive malignancies than those dependent on other oncogenes such as p53 and Ki-ras.[3,7,30] An inverse relationship has been observed between bcl-2 and p53 expression in several malignancies, suggesting that these proteins may interact through opposite mechanisms: inhibition of apoptosis (bcl-2), and promotion of apoptosis (p53).[4,30]

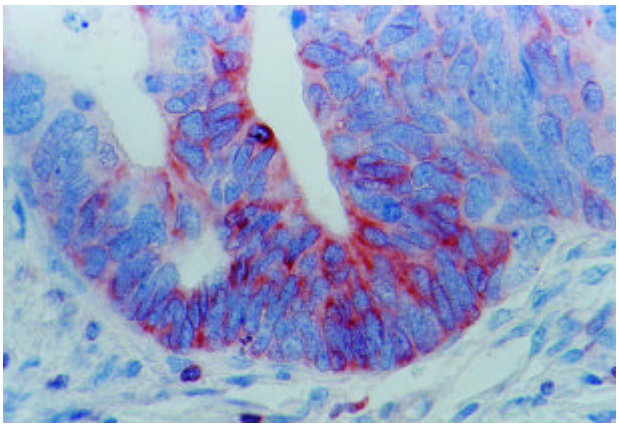

Figure 3. Colonic adenocarcinoma showing cytoplasmic positivity of the tumor cells to bcl-2 protein immmunostain (x100).

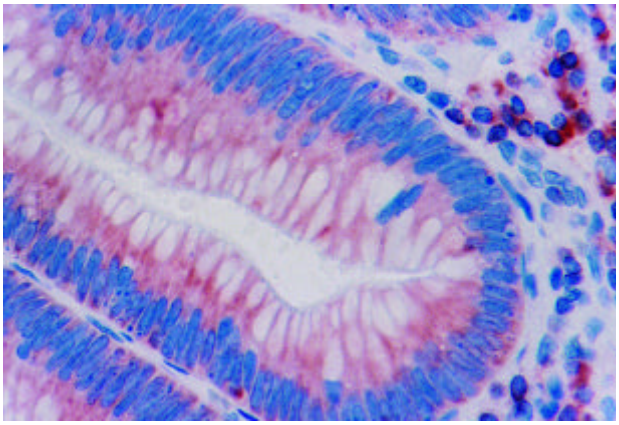

Figure 4. Colonic tubular adenoma displaying cytoplasmic reactivity to bcl-2 immunostain in the adenoma cells. Note also the strong reactivity of the lymphocytes in the lamina propria. (x400).

Bronner et al showed bcl-2 expression in normal gastrointestinal regenerative epithelia compartment and in hyperplastic and neoplastic lesions, but not in reactive or inflammatory conditions.[10] Bcl-2 expression was also noted in the non-dysplastic epithelium closely adjacent to neoplastic lesions, suggesting that altered bcl-2 expression precedes the development of morphologically recognized neoplasia.

In our study, bcl-2 staining was clearly observed in the colonic crypt base with loss of expression in the more superficial compartments. As in previous studies,[5-7,9,10,25,29,31-33] bcl-2 positive staining was more frequently observed in colonic adenomas than in carcinomas. We concur with others that there appears to be a significant decline in bcl-2 expression during the course of tumor progression from adenoma to carcinoma.[1,6,8,25] One hypothesis is that colonic adenoma-to-carcinoma transformation may starts in cells that already express bcl-2, but that selection against continued bcl-2 expression occur because of bcl-2 ability to inhibit apoptosis.

In conclusion, we believe that bcl-2 gene plays a key role in the early stage of colorectal carcinogenesis during evolution from adenoma to carcinoma. Our study shows that bcl-2 expression inversely correlates with Ki67 expression in colorectal carcinoma. Moreover, it appears that bcl-2 IHC expression is associated with favorable prognostic histopathologic features, while Ki67 IHC is associated with unfavorable prognostic pathologic features. Accordingly, bcl-2 and Ki67 immunohistochemical expression should be evaluated prospectively to determine their value in predicting the clinical course of colorectal carcinoma patients. Additional larger studies will help to further clarify the contribution of bcl-2 and Ki67 to the biologic and clinical behavior of colorectal carcinoma.

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