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Prognostic Value of bcl-2 Oncoprotein Expression in Stage II Colon Carcinoma

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Abstract

The bcl-2 proto-oncogene encodes a Mr 25,000 protein that has been shown to prevent apoptosis or programmed cell death. The bcl-2 protein is detectable in basal cells of normal colonic epithelium, and an altered topographic distribution of this protein is found in colonic neoplasms. However, the clinical significance of normal bcl-2 expression in colon carcinomas remains unknown. We examined the prognostic value of the bcl-2 protein in TNM stage II colon carcinomas and its relationship to DNA ploidy, cell proliferation indices, p53 expression, and clinicopathological features. We analyzed 119 resected and otherwise untreated, paraffin-embedded stage II colon carcinomas for bcl-2 and p53 protein expression using immunohistochemistry. DNA ploidy and proliferative index (% S-phase + % G2-M) were determined by flow cytometry, and tumor grade and vascular microinvasion were assessed on histological sections. Cytoplasmic expression of the bcl-2 protein was detected in 72 (66%) of 110 carcinomas, and a high level of expression was significantly correlated with diploid DNA content (P = 0.02) and low proliferative activity (P = 0.005). bcl-2 was not associated with nuclear p53 expression. In a univariate analysis, a higher fraction of bcl-2-positive tumor cells was associated with better relapse-free survival (P = 0.02) and overall survival (P = 0.05) rates. Moreover, a high level of bcl-2 expression was an independent predictor of better relapse-free survival (P = 0.04), but not overall survival (P = 0.14), after adjustment for other variables, including proliferative index, DNA ploidy, and race. In conclusion, bcl-2 overexpression is associated with favorable prognostic features and may predict clinical outcome in stage II colon carcinomas.

Introduction

Colon carcinogenesis is a multistep process characterized by the accumulation of distinct genetic alterations which disorder normal mechanisms controlling cell growth (1, 2). Solid tumor growth is governed by cell production and cell loss, the latter of which can occur by apoptosis or programmed cell death (3, 4). Evidence indicates that apoptosis is regulated by oncoproteins and tumor suppressor genes (5). In this regard, the bcl-2 proto-oncogene has been shown to protect cells from apoptotic cell death in several experimental situations (5, 6). Furthermore, an oncogenic effect of bcl-2 is suggested by data in bcl-2 transgenic mice, wherein lymphocytes overexpressing bcl-2 displayed extended cell survival and progress to high-grade lymphoma (7).

The bcl-2 gene encodes a Mr 25,000 integral membrane protein that localizes to the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (8, 9). Alterations of the bcl-2 gene were first described in follicular and diffuse B-cell lymphomas, wherein its overexpression was shown result from a chromosomal 14;18 translocation (6, 10). The bcl-2 protein has also been detected by immunohistochemical methods in some nonlymphoid tissues, including organized epithelia of the skin, gastrointestinal mucosa, and glandular epithelium (11). In normal epithelia, including colorectal mucosa, bcl-2 protein expression is confined to basal cells corresponding to the location of progenitor or stem cells (10, 12). We have previously reported that the bcl-2 protein is frequently expressed in colorectal dysplastic polyps and carcinomas with an altered topographic distribution compared with normal or hyperplastic epithelia (12). These results and those of others suggest that abnormal activation of the bcl-2 gene is a frequent and early event in colorectal tumorigenesis (12-14).

Studies indicate that wild-type p53 can induce apoptosis in vitro (15), and mutant p53, like bcl-2, can inhibit apoptotic cell death (16). Furthermore, a reciprocal relationship between bcl-2 and p53 expression was found in human breast cancers, suggesting that these oncoproteins may interact in the regulation of apoptosis (17-19). Abnormalities in the p53 tumor suppressor gene are frequent events in colorectal cancers, and some but not all studies have shown p53 protein accumulation to be of prognostic value (20-25). Established prognostic markers in colorectal cancer include tumor stage (26), histological grade (27), vascular microinvasion (28), and in several studies, DNA ploidy status and cell kinetic parameters (29-34). Of note, the relationship of these prognostic features to molecular genetic alterations in colorectal cancer has not been well studied.

Evidence is accumulating to suggest that bcl-2 can influence the clinical behavior of certain human cancers including follicular lymphoma (35), non-small cell lung cancer (36), and breast cancer (17-19). To date, however, the role of bcl-2 in the growth, progression, or prognosis of colorectal cancer is largely unknown. To determine the influence of bcl-2 on clinical outcome, we analyzed bcl-2 protein expression in primary, lymph node-negative colon cancers and related expression to long-term relapse-free and overall survival rates. The relationship between bcl-2 expression and various biological and pathological features was also determined.
Materials and Methods

Patient Materials. The study population included 119 patients with primary colon adenocarcinomas who were treated surgically at the University of Chicago Medical Center between 1965 and 1987. Patients with rectal carcinoma were excluded from this study. The primary tumor had been formalin-fixed and paraffin-embedded and consecutive 4–6-μm-thick sections were cut from the respective tumor blocks. All tumors were staged according to the American Joint Committee on Cancer (37) and all were stage II cancers (invasion of the muscularis propria (T2, 10 cases) or serosal extension/penetration (T3/T4a, 109 cases)) without lymph node metastasis. Patients did not receive postoperative systemic chemotherapy until the development of metastatic disease. Patient specimens were examined from 61 men and 58 women including 68 whites and 51 blacks. The ages of the patients ranged from 26 to 89 years, with a median age of 68 years (mean age, 65.6 years; SD = 11.5). Sixty-nine tumors were left-sided and 50 were right-sided, with the reference point being the splenic flexure. The median postoperative follow-up time was 121 (range, 2–281) months.

Immunohistochemistry. An immunoperoxidase method employing an avidin-biotinylated horseradish peroxidase complex (Vectorstain Elite ABC; Vector Laboratories, Burlingame, CA) was used to detect bcl-2 and p53 proteins in deparaffinized tissue sections as described previously (12). Antigen recognition was facilitated by microwave treatment of the slides. The primary anti-bcl-2 mAb (Dako Corporation, Carpenteria, CA) is a murine antihuman mAb, subclass IgG1, that recognizes a cytoplasmic epitope of bcl-2 (38). The anti-p53 mAb DO1 (Onco-gene Science, Inc., Manhasset, NY) is a murine antihuman mAb, subclass IgG2a, that recognizes an amino terminal epitope of the human p53 molecule. For both mAbs, staining was developed by immersing slides in 3.3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO), and the slides were counterstained in hematoxylin (Richard Allen, Richland, WA).

Formalin-fixed, paraffin-embedded sections of normal human lymph node served as a positive control for bcl-2, and a colon cancer known to possess nuclear p53 staining in 75% of cells of adjacent normal mucosa, when present, which also served as an internal positive control and were arbitrarily designated as p53+. For p53, intensity was judged relative to an intensely stained (3+) p53-positive control colon neoplasm. The primary antibody, but included all other steps of the procedure. Negative control slides did not exhibit immunostaining.

Immunostained slides were examined independently by two observers (F. S. and J. H.) who were blinded to clinical outcome. When different interpretations existed, a consensus result was reached after reexamination. Anti-bcl-2 and p53 immunostaining were evaluated and scored separately. The intensity of bcl-2 and p53 immunostaining was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. Percent-age of positive tumor cells was quantified and assigned to one of five categories: 0, ≤5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, ≥75%. For both oncoproteins, ≤5% positive cells were used as the cutoff to define negative tumors. For bcl-2, intensity was judged relative to infiltrating lymphocytes or basal epithelial cells of adjacent normal mucosa, when present, which also served as internal positive controls and were arbitrarily designated as 3+. For p53, intensity was judged relative to an intensely stained (3+) p53-positive control colon neoplasm.

Histopathology. The original slides for each patient were reviewed to confirm the pathological stage and histological grade. The presence or absence of vascular microinvasion was also determined on hematoxylin and eosin-stained sections from the same block of each case. The pathological features were determined independently of immunostaining results. Vessel and/or lymphatic microinvasion was defined as the presence of tumor cell(s) within an endothelial-lined space.

DNA Ploidy. Fifty-μm sections from the same paraffin blocks used for immunohistochemistry were obtained for flow cytometry. Samples were incubated overnight in propidium iodide solution and were analyzed as previously described (39). The DI was calculated as the ratio of the modal value of the DNA histogram of the tumor sample to that of the reference cells. By definition, diploid tumors have a DI of 1.0, and tumors with a single G0/G1 peak at 2C were regarded as diploid. Samples with a second peak distinct from the diploid 2C peak and with a corresponding 4C peak were considered to be aneuploid. In practice, tumors with a distinct G0/G1 population and with a DNA index over 1.18 were classified as aneuploid (40); those with a DI of 1.9–2.1 were classified as tetraploid. Given that conflicting reports exist regarding the clinical behavior of tetraploid tumors (29, 31), these cases were classified as aneuploid but also analyzed separately without a significant change in results. Cell proliferation measurements were performed on DNA histograms from all tumors. PI was calculated as the sum of the percentages of cells in S-phase and G2-M phases of the cell cycle. An estimation of the S-phase fraction was computed as the percentage of cells with a DNA content between 2.5 and 3.8 C.

Clinical Follow-Up. The disease status of each patient was determined at the date of the last follow-up. Follow-up data were gathered by chart review and prior yearly contacts with the patient or treating physician. Patients were classified as having (a) no evidence of disease, (b) local/regional recurrence, or (c) metastatic disease. Disease relapse was defined as either local recurrence or the development of distant metastasis and was determined by clinical and radiographic studies or by surgical means in all cases. The vital status of the patients was also determined at the date of last follow-up and if deceased, cause of death was ascertained from the medical record and/or death certificate. Cause of death was classified as secondary to or unrelated to colon carcinoma. Only colon cancer deaths were considered as outcome events given the extended patient follow-up time.

Statistical Analysis. We considered both staining intensity and percentage of positive tumor cells in all analyses and grouped the data to evaluate the prognostic role of bcl-2 and p53 expression. Various cutoffs were evaluated to define low versus high oncoprotein expression. For bcl-2, 50% bcl-2-positive cells...
was found to give the best separation between the subgroups at low and high risk of disease relapse and colon-specific death. With regard to p53, 25% p53-positive cells was found to best separate patients into low- and high-risk groups. The associations between groups were analyzed using the $\chi^2$ test. The primary statistical outcomes were RFS and OS rates measured from the date of surgery. Both the time to relapse and to colon-specific death were analyzed using the Cox proportional hazards model for the univariate and multivariate analyses (41). All potential prognostic factors were entered into a stepwise regression model from which significance levels were determined. All important prognostic factors, as shown in a univariate analysis, as well as $bcl-2$ expression and covariates correlated with $bcl-2$, were included in the multivariate model to assess their predictive effects on RFS and OS rates. Additionally, the risk ratios for prognostic variables and their 95% confidence intervals were computed. RFS and OS data were plotted using the Kaplan-Meier method. Statistical significance was defined as a two-sided $P$ value $\leq 0.05$.

Results

Immunohistochemistry. Cytoplasmic staining for the $bcl-2$ protein was observed in 72 (66%) of 110 carcinomas (Fig. 1, C and D). Immunoreactivity was generally most prominent in the apical portion of the tumor cells and also frequently involved the nuclear membrane. Staining localized to carcinoma cells in malignant glands of the parabasal and superficial regions of the tumors (12). This was in contrast to histologically normal mucosa from the resection margins wherein $bcl-2$ staining was restricted to basal epithelial cells of the colonic crypts as described previously (11, 12; Fig. 1A). Among $bcl-2$-positive cases, at least 50% of carcinoma cells stained positively in 37 (51%) of 72 tumors. Strong (3+) staining of the cytoplasm of lymphocytes in the lamina propria was observed in normal sections (Fig. 1, A and B) and in infiltrating lymphocytes within tumor stroma.

Consistent with other studies (18–23), nuclear p53 staining was found in 56 (48%) of 117 colon carcinomas. Nuclear p53 staining was detected in at least 50% of tumor cells in 44 (79%) of 56 cases with positive staining. Normal colonic mucosa was uniformly negative for the p53 protein.

Clinicopathological Features. The degree of tumor differentiation was as follows: 32 well, 80 moderate, and 7 poor. Vascular microinvasion was present in 15 (13%) of 119 cases. No relationship between $bcl-2$ expression and either of these features was observed. Furthermore, no relationship between the level of $bcl-2$ expression and patient age, sex, race, or tumor location was found (Table 1). No association between $bcl-2$ and p53 protein expression was detected. Nuclear p53 protein expression was significantly associated with distal as opposed to proximal colon carcinomas ($P = 0.001$). No association between p53 expression and histological grade, microinvasion, or race was observed.

DNA Ploidy and Cell Proliferation. Analysis of DNA content revealed that 56 tumors were diploid and 61 were aneuploid. A significantly higher level of $bcl-2$-positive tumor cells was found in diploid versus aneuploid carcinomas (54% versus 34%; $P = 0.02$; Table 1). A PI cutoff of 15% or greater gave the best separation between the subgroup at high- versus low-risk (<15%) of relapse and death. The mean PI (% S-phase plus % G2-M) was 17.2 ± 8.5 (median, 14.3; range, 3.9–35.6). A reciprocal relationship between $bcl-2$ expression and proliferative activity was detected. In this regard, a high level of $bcl-2$ expression was significantly more common in tumors with low

<table>
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<td>Deaths, colon cancer 21</td>
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<td>Mo. 121</td>
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<td></td>
<td>Range (2–281)</td>
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</table>

* Percentage of $bcl-2$-positive tumor cells: less than 50% equals low, at least 50% equals high. 9 cases, insufficient material.

a American Joint Committee on Cancer and the TNM Project of the UICC, 1987 (37).

d Refers to tumor location relative to the splenic flexure.

e Percentage of p53-positive tumor cells: less than 25% equals low, at least 25% equals high.

f PI equals percentage of tumor cells in S-phase plus G2-M.

g 2 cases, not analyzed.

Table 1 Clinicopathological characteristics of patients with stage II colon carcinoma as a function of $bcl-2$ expression

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bcl-2 Expression in Stage II Colon Carcinoma

(A) and (D) show similar patterns of bcl-2 expression in Stage II Colon Carcinoma. (B) and (C) demonstrate different expression patterns.
proliferation (60%) than in those with high proliferation (33%, \( P = 0.005 \)).

Nuclear p53 expression was significantly more frequent in aneuploid versus diploid tumors (71% versus 38%; \( P < 0.001 \)) and in those with a PI \( \geq 15\% \) (63% versus 40%; \( P = 0.01 \)).

**Clinical Outcome.** At a median follow-up of 121 months, disease relapse occurred in 24 (20%) of 119 patients with 6 local/regional recurrences and 18 distant metastases. There were 21 (18%) colon cancer deaths and 53 deaths from causes unrelated to colon cancer (Table 1). Disease relapse was significantly more common in patients with low (less than 50%) bcl-2-positive cells compared with high bcl-2 expression (28.8\% versus 9.8\%; \( P = 0.04 \)). In a univariate analysis, a higher fraction (at least 50%) of bcl-2-positive tumor cells was a significant predictor of better RFS (\( P = 0.02 \)) and disease-specific OS (\( P = 0.05 \)) rates (Table 2 and Fig. 2). Similarly, a low PI (<15\%) was also a significant predictor of better RFS (\( P = 0.005 \)) and OS (\( P = 0.005 \)). The poorest outcome was observed in tumors with a low level of bcl-2 expression and high proliferation rates. Diploid tumors had more favorable RFS (\( P = 0.06 \)) and OS (\( P = 0.07 \)) rates and when ploidy was combined with proliferative activity, the combined parameter was a significant predictor of RFS (\( P = 0.02 \)) and OS (\( P = 0.02 \)) rates.

p53 protein expression failed to predict RFS (\( P = 0.51 \)) or OS (\( P = 0.38 \)) (Table 2). Clinicopathological features associated with better RFS were age (\( P = 0.03 \)) and race, i.e., nonblack (\( P = 0.02 \)), however, only the latter predicted overall survival (\( P = 0.04 \)). Histological grade failed to predict clinical outcome. However, when the data were grouped, i.e., well plus moderate versus poor differentiation, better RFS (\( P = 0.09 \)) and OS (\( P = 0.04 \)) rates were observed for increased tumor differentiation (data not shown). When patient outcome was analyzed according to tumor location or vascular microinvasion, no significant relationships were observed. Analysis of \( T_3 \) and \( T_4 \) (Dukes’ stage B2) only patients, thereby omitting the nine \( T_2 \) (Dukes’ stage B1) tumors, did not significantly alter the results.

Multivariate analysis was performed according to the Cox proportional hazards model to evaluate the prognostic value of bcl-2 expression after adjustment for other prognostic factors and variables correlated with bcl-2 (Table 3). Our results indicate that a higher fraction (\( \geq 50\% \)) of bcl-2-positive tumor cells was associated with a significantly better RFS (\( P = 0.04 \)) after adjusting for the effects of DNA ploidy, PI, and race. However, bcl-2 failed to maintain its prognostic role for OS (\( P = 0.14 \)). Low (\( \leq 15\% \)) versus high PI was an independent predictor of more favorable RFS (\( P = 0.03 \)) and OS (\( P = 0.02 \)) rates. When the PI was omitted from the model, bcl-2 expression was found to significantly predict RFS (\( P = 0.03 \)) but not OS (\( P = 0.09 \)) rates.

**Discussion**

The present study was undertaken to evaluate the prognostic role of the bcl-2 protein and its relationship to biological and pathological features in colon carcinomas. Given that 20–30\% of patients with stage II colon carcinomas will eventually relapse and die of their disease (26), the identification of stage-independent prognostic variables would alter therapeutic decision making in that effective adjuvant therapy is available (42). Our results indicate that abnormal bcl-2 protein expression is detectable in a high percentage (66\%) of stage II colon carcinomas. A loss of topographic restriction in bcl-2 staining was found in carcinomas, compared with normal colonic mucosa, as previously reported by ourselves (12) and others (13).

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**Figure 1** A and B, normal colonic mucosa immunostained for the bcl-2 protein demonstrates cytoplasmic bcl-2 staining (brown reaction product) restricted to basal epithelial cells of the colonic crypts, corresponding to stem cells. bcl-2 staining is also seen in some lymphocytes within the lamina propria. C and D, colonic adenocarcinoma with strong (3+) cytoplasmic bcl-2 staining in carcinoma cells of the malignant glands. Complete loss of topographic restriction is seen compared to normal mucosa. Immunoperoxidase stain, diaminobenzidine chromagen; A, \( \times 50 \); B, \( \times 100 \); C and D, \( \times 250 \).
bcl-2 Expression in Stage II Colon Carcinoma

Fig. 2 Probability of long-term RFS (A) and overall disease-specific survival (B) in patients with stage II colon carcinoma as a function of bcl-2 expression. High bcl-2 refers to tumors with 50% or more bcl-2-positive cells; low bcl-2 refers to tumors with less than 50% bcl-2-positive cells. P values result from the univariate analysis as shown in Table 2.

Our results represent the first data on the prognostic value of bcl-2 in colon cancer. We found that despite the relatively small number of patients with tumor recurrence (20%), a higher fraction of bcl-2-positive tumor cells was a significant predictor of RFS according to univariate and multivariate analyses. bcl-2 expression, however, did not maintain its prognostic role with regard to overall survival when adjusted for other features, mainly PI, which was the strongest prognostic variable. In clinical studies in colorectal cancer, RFS data are generally more sensitive than overall survival data given the advanced age of the patients at diagnosis (mean age at surgery in this series, 65.6 years) and the increased number of deaths unrelated to colorectal cancer during the follow-up period.

**Table 3** Multivariate analysis of long-term RFS and OS in patients with stage II colon carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>RFS Risk ratio (95% CI)</th>
<th>P</th>
<th>OS Risk ratio (95% CI)</th>
<th>P</th>
</tr>
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<td>bcl-2 Expression</td>
<td>0.35 (0.12-0.98)</td>
<td>0.04</td>
<td>0.45 (0.16-1.29)</td>
<td>0.14</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>0.53 (0.16-1.77)</td>
<td>0.30</td>
<td>0.49 (0.13-1.75)</td>
<td>0.27</td>
</tr>
<tr>
<td>PI</td>
<td>4.50 (1.17-17.3)</td>
<td>0.03</td>
<td>5.89 (1.36-25.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>Race</td>
<td>1.96 (0.83-4.64)</td>
<td>0.12</td>
<td>1.77 (0.71-4.46)</td>
<td>0.22</td>
</tr>
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</table>

*For reference groups see Table 2.  
CI, confidence interval.

An association between bcl-2 expression and a more favorable clinical outcome has also been reported in patients with breast cancer (17–19), non-Hodgkin’s lymphoma (35), and non-small cell lung cancer (36). Consistent with studies in breast cancer (17, 18), we found that a higher fraction of bcl-2-positive tumor cells was significantly associated with diploid DNA content and low proliferative activity, including the S-phase fraction. Increased bcl-2 expression may therefore be associated with slower neoplastic growth rates, thereby contributing to a better prognosis (11). Interestingly, an *in vitro* study has shown that transfection of the bcl-2 gene into several solid tumor cell lines, including those derived from human colon carcinomas (43), inhibits their growth. Additionally, the bcl-2 protein interacts with other members of the bcl-2 family, including Bax, which may also influence its function (5, 44).

DNA aneuploidy reflects net changes in chromosomal number; however, the molecular genetic abnormalities producing aneuploidy remain unknown. Previous studies have shown that aneuploidy is associated with increased p53 expression (45) and fractional allelic loss on chromosomes 17p and 18q (46). We found increased p53 expression and low bcl-2 to significantly correlate with aneuploidy and increased PI, i.e., S-phase plus G2-M. Therefore, the function of the bcl-2 and p53 oncogenes may influence or reflect these biological processes. The prognostic significance of aneuploidy in colorectal cancer remains controversial with some, but not all, studies showing an adverse effect on clinical outcome (29–34). However, our findings and those of others indicate that the PI is an independent prognostic marker that may be a more powerful predictor of adverse outcome than aneuploidy (32–34, 47).

An inverse relationship between bcl-2 and p53 expression has been reported in breast cancers (17–19) and in non-Hodgkin’s lymphoma (48), suggesting that these oncogenes may regulate a common cell death pathway. We did not identify an association between bcl-2 and p53 protein expression in these colon cancers. However, we previously reported a reciprocal regulation of apoptosis (5), the association between bcl-2 and p53 may be tumor specific or possibly related to the stage of neoplastic development. Alternatively, an interrelationship between these oncogenes may exist in colorectal cancers that is not detectable by a simple comparison of immunostaining results.

Consistent with other studies (21, 22, 49, 50), we found nuclear p53 expression in a higher percentage of distal versus...
proximal colon cancers. Although mutant p53 was associated with aneuploidy and increased proliferation, its expression did not predict RFS or OS rates in the entire series nor when analyzed according to tumor location. Given that p53 protein expression has been associated with reduced survival in some, but not all, series (20–25), its prognostic value in colorectal cancer remains controversial. Discordant immunostaining results may result from use of different antibodies, scoring systems, fixation conditions, failure to account for tumor stage, and recognition of wild-type p53 protein. Furthermore, immunostaining identifies only the most common mutations, i.e., missense mutations which induce synthesis of mutant p53 proteins (50). Nonsense or splicing p53 mutations and allelic loss on chromosome 17p would thereby go undetected. These findings explain, in part, why p53 gene mutations detected by immunohistochemistry versus single-strand conformation polymorphism analysis are concordant in only 69% of colorectal cancers (51).

In the present study, patients were treated with surgery alone and did not receive chemotherapy until the development of metastatic disease. Studies have shown that cells expressing either bcl-2 or mutant p53 are resistant to inducers of apoptosis, including radiation and several DNA-damaging anticancer drugs, including fluorouracil (52–55). Therefore, expression of the bcl-2 and p53 oncoproteins in colon carcinomas may contribute to their relative resistance to anticancer therapies. To address this issue, studies correlating bcl-2 and p53 protein expression in colon and rectal carcinomas with response to chemotherapy or chemoradiation are in progress in our laboratory. In conclusion, bcl-2 overexpression is a frequent and abnormal event in colon carcinomas and is associated with favorable prognostic features. bcl-2 expression can predict clinical outcome in stage II colon cancers, although prospective studies are needed to validate these findings.

References

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Prognostic value of bcl-2 oncoprotein expression in stage II colon carcinoma.

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