Loss of Bcl-2 expression correlates with tumour recurrence in colorectal cancer

M Ilyas, X-P Hao, K Wilkinson, I P M Tomlinson, A M Abbasi, A Forbes, W F Bodmer, I C Talbot

Abstract

***Aims*—To investigate the association between immunohistochemical expression of Bcl-2 and p53 in colorectal cancer and tumour recurrence following surgery.**

***Methods*—Sixty six cases of Dukes' B colorectal carcinoma were studied. All tumours were moderately differentiated and were shown to be histologically clear of the resection margins. Immunohistochemistry was performed on formalin fixed paraffin wax embedded tissue using monoclonal antibodies for p53 and Bcl-2. The Bcl-2 staining was assessed separately for relative intensity of staining and percentage of positive tumour cells and given a final score which combined the two factors. The p53 staining was assessed on number of positive tumour cells only. The patterns of immunostaining of those cases in which there had been tumour recurrence were compared with those cases in which there was no tumour recurrence (controls).**

***Results*—A statistically significant inverse association was found between Bcl-2 score and tumour recurrence (median Bcl-2 score of 6 (interquartile range (IQR) 2–9) in patients with recurrent disease; median Bcl-2 score of 8 (IQR 6–10) in those without recurrence; p=0.03). When examined separately, both the intensity of expression and percentage of positive tumour cells were significantly associated with tumour recurrence (p=0.04 in each case). There was no association between p53 staining and tumour recurrence.**

***Conclusion*—Results suggest that, when controlled for differentiation, Bcl-2 expression is a prognostic marker and may be useful as an adjunctive test in clinical decision making.**

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Keywords: colorectal cancer; tumour recurrence; Bcl-2; p53; Jass grouping

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Colorectal cancer (CRC) is the third most common cause of cancer related death in the Western world.[1] The prognosis in patients who develop colorectal cancer is dependent on Dukes' staging and Jass grouping at the time of surgery.[2] Tumour grade provides further information although, on the whole, tumour grade parallels tumour stage and poorly differentiated tumours tend to be relatively advanced. A significant proportion of patients present with Dukes' stage B tumours. These patients have an intermediate prognosis with a five year survival of approximately 70%. Given the increase in the number of adjuvant treatments available it would be advantageous to be able to subclassify this group of tumours into those which have a good outlook and therefore probably do not need adjuvant therapy, as opposed to those with graver outlook, in which case the patients may benefit from adjuvant therapy.

Bcl-2 is a cytoplasmic protein which localises to mitochondria, endoplasmic reticulum, and the nuclear envelope. The protein can be identified in many different tissues and is thought to have a role in the inhibition of apoptosis.[3 4] In follicular lymphomas, for example, the translocation t(14;18) results in constitutive overexpression of Bcl-2 and immortalisation of lymphocytes.[5 6] Similarly, Bcl-2 overexpression has been identified in a large number of epithelial tumours in which its role as an inhibitor of apoptosis is thought to promote tumour growth.[7–12] In tumours arising from the large intestine, Bcl-2 overexpression has been reported in a high proportion of adenomas including unicryptal adenomas.[8 13 14] Colorectal cancers have also been reported to show Bcl-2 overexpression although, in comparison with adenomas, there is lower intensity of expression in the invasive tumours.[8 13 14] There may also be a loss of expression with loss of tumour differentiation[15] and it would appear that the role of Bcl-2 is probably more important in the early development of colorectal tumours than in later tumour progression.

p53 is a nuclear oncosuppressor protein which is involved in the maintenance of genomic integrity: DNA damage results in the increased expression of p53 which then causes G1 arrest in actively cycling cells.[16] It can then induce factors which allow DNA repair to occur or, if the damage is too great, it can induce factors which cause apoptosis. p53 mutation occurs as a late event in the transition from an adenoma to an invasive carcinoma[17] and is easily detected by immunohistochemistry due to stabilisation of the mutated p53 protein.[18]

Several studies have examined the correlation of p53 overexpression and prognosis in colorectal cancer with mixed results reported in the literature.[19–23] In one study of Bcl-2 expression, high Bcl-2 expression was shown to correlate with a good outcome.[15] We aimed to examine the comparative value of immunohistochemical Bcl-2 and p53 expression as predictors of tumour recurrence. In order to control for differentiation and Dukes' staging, we only examined moderately differentiated Dukes' B tumours.

Table 1 Patient characteristics

	Non-recurrence	*Recurrence*
Total	51	15
Male	25 (49.0%)	10 (66.7%)
Mean (SD) age (y)	61.0 (11.5)	62.1 (11.6)
Jass		
I	11 (21.6%)	0
II	29 (26.9%)	8 (53.3%)
III	11 (21.6%)	7 (46.7%)
Median Bcl-2 (IQR)	8 (6–10)	6 (2–9)

IQR, interquartile range.

Materials and methods

CASE SELECTION

Sixty six Dukes' stage B colorectal cancers were retrieved from the archives of St Mark's Hospital. All patients had been followed up for at least six years. The group consisted of 15 cases which had developed tumour recurrence within six years and 51 controls who were recurrence-free at six years. All operations had been performed at St Mark's Hospital and were considered curative operations. The age distribution of patients with recurrent tumours was similar to that of control patients although a higher proportion were male (table 1). Of those patients with tumour recurrence, 11 had systemic metastasis and four had local recurrence only (either pelvic recurrence for rectal tumours or peritoneal recurrence for colonic tumours).

Histological examination had shown that the tumours were clear of all resection margins and the radial margin. In a previous review of these tumours, we have shown them all to be moderately differentiated and we have assessed them for Jass grouping and E cadherin expression.[24]

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Immunohistochemistry was performed on sections from formalin fixed paraffin wax embedded tumour tissue using the labelled streptavidin method. Fresh 4 μm thick sections were dewaxed in xylene and rehydrated in graded alcohols. These were incubated for 20 minutes in 0.5% hydrogen peroxide in methanol in order to block endogenous peroxidase activity. Both antibodies need a step for antigen retrieval which was achieved by heating the sections for 30 minutes in an aluminium pressure cooker at 15 psi in sodium citrate buffer (0.01M, pH 6.0). Sections were then incubated overnight at room temperature with mouse monoclonal antibodies for Bcl-2 (Dako) and p53 (DO-7, Novocastra) at a dilution of 1/40 and 1/100 respectively. A second layer of biotinylated polyclonal rabbit antimouse antibody (Dako) was applied for one hour (in a dilution 1/200) followed by a final layer of horseradish peroxidase labelled streptavidin (Dako) for one hour (in a dilution of 1/200). The bound antibody was detected with diaminobenzidine as the chromogen. Negative controls (performed using phosphate buffered saline (PBS) buffer instead of the primary antibody) and positive controls (consisting of tissue known to be positive for Bcl-2 and p53) were included in every experiment.

Results of the immunohistochemistry were reviewed independently by two pathologists (MI and X-PH) who were blinded as to the outcome of the tumours. Given the heterogeneity of the Bcl-2 staining, a previously described dual scoring system giving equal weight to both intensity of staining and extent of staining of tumour cells was used.[25] Intensity was scored on a scale of 0–3 with 3 being equivalent to the intensity seen in the proliferating cells in the base of crypts. Extent of staining of each tumour was scored semiquantitatively from 0 to 3 using the following scale: 0 = no staining at all in the tumour; 1 = less than 25% of cells; 2 = 25–50%; 3 = more than 50%. The scores of both reviewers were then added together to give a score between 0 and 12. For p53 immunostaining, tumours were scored in a semiquantitative manner using the following scale: 0 = less than 10% positive cells; + = 10–25%; ++ = 25–50%; +++ = more than 50%. The results of the two reviewers were compared and any tumours in which there was not agreement were reviewed together by both pathologists and a consensus was reached.

DATA ANALYSIS AND STATISTICS

The results of the immunostaining for each tumour were analysed by KW and IPMT. Only they had access to data regarding the outcome of each patient and at no stage was either of the pathologists given access to these data. The Wilcoxon rank sum test was used to examine the relation between Bcl-2 score and tumour recurrence and between Bcl-2 score and p53 expression. Categorical variables were compared using a χ^2 statistic with tests for linear trend where appropriate. The relation between Bcl-2 and recurrence adjusted for age, sex, Jass group, and p53 expression was examined using multiple logistic regression. Improvements in model fit were based on likelihood ratios. In this model, Bcl-2 score, Jass group, and age were fitted as untransformed numerical values, while p53 was fitted as a binary variable (negative or positive for overexpression). Analyses were carried out for the whole group and for the subset created by excluding patients having only local recurrence.

Results

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Normal epithelium showed strong staining for Bcl-2 at the crypt bases with a gradient of diminishing intensity of staining along the crypt axis (fig 1). Lymphocytes in the stroma and Peyer's patches stained with equal intensity to that of the epithelial cells in the bases of the crypts and served as internal positive controls. Staining of the tumours was generally heterogeneous, ranging from complete negative staining (intensity 0, extent 0) (fig 2A) through all degrees of intermediate staining (for example, extensive pale staining: intensity 1, extent 3 (fig 2B); and focal intense staining: intensity 3, extent 1 (fig 2C)) to extensive intense staining (intensity 3, extent 3 (fig 2D)).

The p53 immunohistochemistry showed strong staining in the tumour nuclei (fig 3). A case was regarded as positive for p53 overexpression if there was staining of over 10% of the tumour cells. There were occasional cases in which there was cytoplasmic staining but no

Figure 1 Bcl-2 expression in non-neoplastic mucosa. There is intense Bcl-2 expression in the base of the crypts with a gradient of diminishing intensity along the crypt axis. The lymphocytes of the lamina propria are also positive and act as internal positive controls.

nuclear staining. These were regarded as negative since the meaning of cytoplasmic localisation of p53 in colorectal tumours is uncertain.

DATA ANALYSIS

Figure 4 is a scatter plot of the Bcl-2 scores for all the tumours. Scores for Bcl-2 staining were significantly lower in patients with recurrent tumours than in controls. The median score in patients with recurrent tumours was 6 (interquartile range (IQR) 2–9) compared with a median of 8 (IQR 6–10) in controls (p=0.03, Wilcoxon rank sum test). Similar differences between recurrent and non-recurrent groups were seen for the intensity of Bcl-2 staining and for the percentage of positive cells when these were analysed separately (p=0.04 in each case). The median score for the tumours overall was 8 and, using this as a cut off point, we classed the tumours into a group showing "high" Bcl-2 score (8 or more) and "low" Bcl-2 (7 or less). Thirty five of 51 (69%) non-recurrent tumours showed a high Bcl-2 score compared with five of 15 (33%) of the recurrent tumours. This is a significant association between high Bcl-2 expression and freedom from tumour recurrence (χ^2_1=6.04, p=0.014).

Furthermore, if Bcl-2 staining was categorised into four groups (table 2) there was evidence of a linear trend of decreasing risk of tumour recurrence at higher Bcl-2 scores (p=0.01). The result was similar if cases having only local recurrence were excluded (n=62, p=0.01).

In the study group as a whole, Jass scores were higher in patients with a recurrent tumour than in controls (table 1; χ^2_3=5.9, p=0.05). Twenty nine of 51 (57%) non-recurrent tumours and eight of 15 (53%) recurrent tumours were positive for p53 overexpression (table 3) showing that there is no association between p53 expression and tumour recurrence (χ^2_3=5.3, p=0.15). There was no association between p53 positivity and Bcl-2 score (p= 0.47, Wilcoxon rank sum test) or between

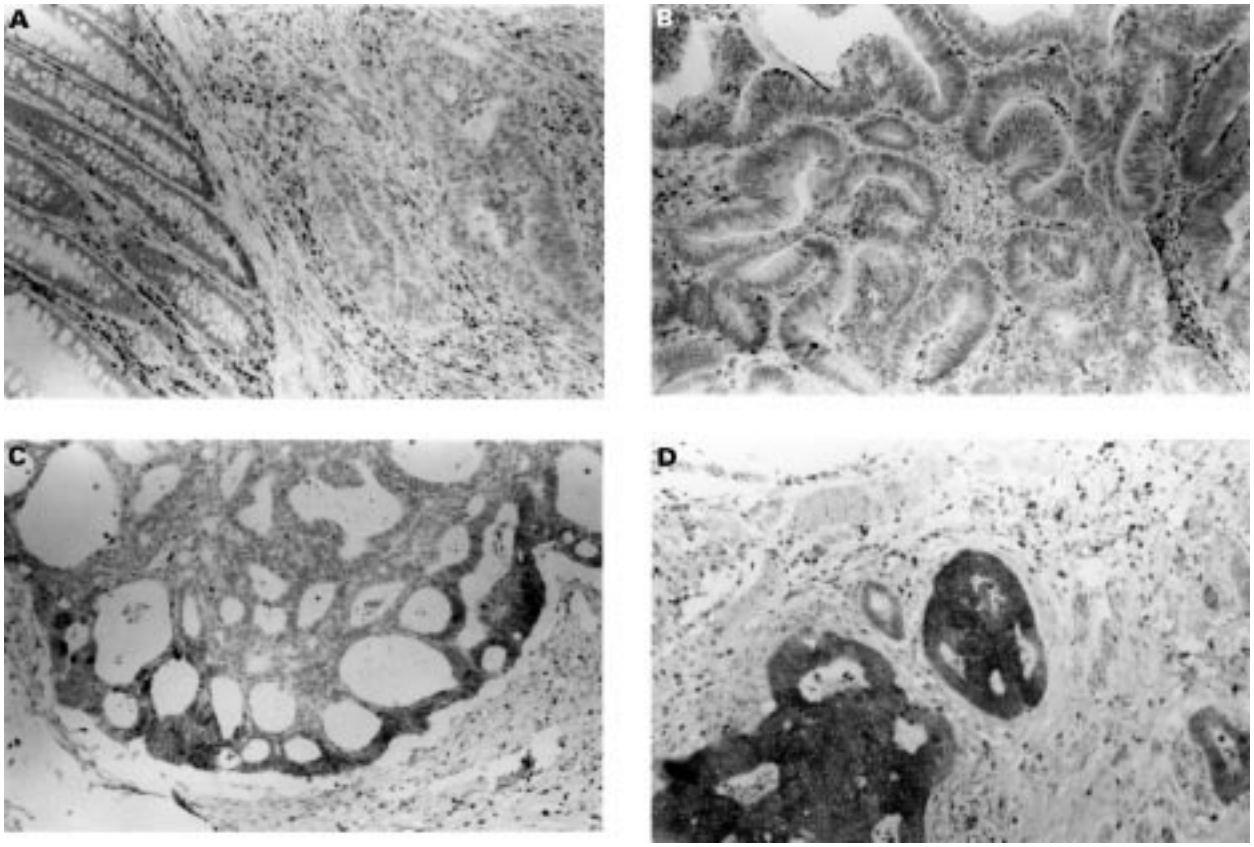

Figure 2 Heterogeneous expression of Bcl-2 in colorectal tumours. (A) Tumour which is negative for Bcl-2 expression while the peritumoural lymphocytes and adjacent mucosa can be seen to be positive. (B) Tumour showing weak but unequivocal expression in most tumour cells. (C) Tumour which is predominantly negative except for focal intense expression. (D) Intense expression can be seen in most tumour cells.

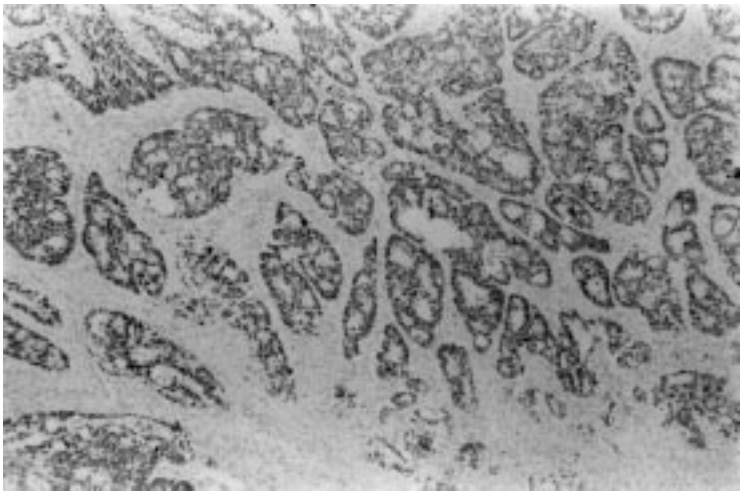

Figure 3 p53 expression in a colorectal tumour, showing strong nuclear expression in most tumour cells.

Table 2 Relation between Bcl-2 score and recurrence

Bcl-2	*Non-recurrent*	*Recurrent*
All patients (n=66)		
0–3	4 (50.0%)	4 (50.0%)
4–6	10 (62.5%)	6 (37.5%)
7–9	17 (89.5%)	2 (10.5%)
10–12	20 (87.0%)	3 (13.0%)
Distant metastases if recurrent (n=62)		
0–3	4 (50.0%)	4 (50.0%)
4–6	10 (76.9%)	3 (23.1%)
7–9	17 (89.5%)	2 (10.5%)
10–12	20 (90.9%)	2 (9.1%)

All patients: test for heterogeneity, $\chi^2_3 = 8.22$, p = 0.04; test for linear trend $\chi^2_1 = 6.45$, p = 0.01.
Distant metastases: test for heterogeneity $\chi^2_3 = 7.76$, p=0.05; test for linear trend $\chi^2_1 = 6.13$, p=0.01.

Table 3 Relation of p53 staining with tumour recurrence

p53 score	*Non-recurrence*	*Recurrence*	*Total*
–	22	7	29
+	2	3	5
++	10	1	11
+++	17	4	21
Total	51	15	66

$\chi^2_3 = 5.3$, p > 0.1.
–, < 10% positive tumour cells; +, 10–25% positive tumour cells; ++, 26–50% positive tumour cells; +++, > 50% positive tumour cells.

Table 4 Logistic regression model

Variable	*Odds ratio*	*95% Confidence intervals*	*p Value*
All patients (n=66)			
Bcl-2	0.77	(0.62–0.96)	0.02
Age	1.03	(0.97–1.09)	0.35
Sex (F)	0.44	(0.10–1.87)	0.27
Jass	6.49	(1.66–25.38)	0.01
p53 (positive)	0.53	(0.13–2.19)	0.38
Distant metastases if recurrent (n=62)			
Bcl-2	0.79	(0.62–1.00)	0.05
Age	1.05	(0.97–1.13)	0.20
Sex (F)	0.19	(0.03–1.28)	0.09
Jass	5.50	(1.12–27.12)	0.04
p53 (positive)	0.44	(0.08–2.45)	0.35

Jass grouping and Bcl-2 score (data not shown).

To assess the association of Bcl-2 with tumour recurrence independently of Jass score and p53, a logistic regression model was used (table 4). This suggested that Bcl-2 score was significantly associated (p=0.02) with recurrence after controlling for age, sex, Jass group, and p53 score. After excluding patients with purely local recurrence, the corresponding p value was 0.05.

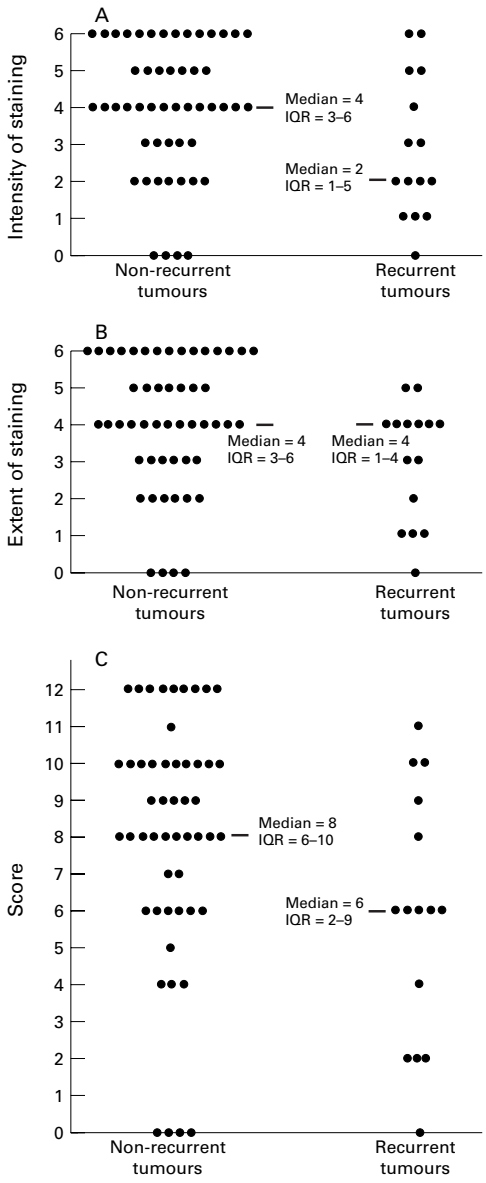

Figure 4 Scatter plots of Bcl-2 scores for (A) intensity, (B) percentage positive tumour cells, and (C) combined scores in the cancers of those patients in whom the tumours eventually recurred and those who remain free from recurrence. IQR, interquartile range.

Discussion

In this study we investigated the usefulness of changes in Bcl-2 and p53 expression as prognostic factors in colorectal carcinoma. We have previously shown, in this group of tumours, that recurrence is correlated with Jass grouping but not changes in E cadherin expression. Data presented provide evidence for an inverse association between Bcl-2 score and risk of tumour recurrence. In contrast, no significant difference in p53 overexpression was seen between the recurrent and non-recurrent tumours.

Other studies have shown that loss of Bcl-2 expression correlates with poor prognosis in both colorectal and non-colorectal

tumours.[9 10 15] These studies have not been controlled for tumour differentiation and tumour stage but have shown, on the whole, that there is loss of Bcl-2 expression with loss of tumour differentiation. Our study was controlled for both of these factors and showed that, in invasive colorectal tumours, Bcl-2 expression is an independent prognostic marker. If these results are corroborated by other studies, then immunohistochemical expression of Bcl-2 may make a useful contribution to clinical management. Cancers presenting at Dukes' B stage form a heterogeneous group and it may be possible to separate those cases which have a higher risk of recurrence, and may therefore need adjuvant therapy, from those cases in which surgery alone is likely to be curative. That there is no correlation between p53 expression and prognosis is in agreement with some studies but not others. It may be that our study was too small and a larger study may have shown some prognostic value of p53 immunostaining.

This study also showed that Jass grouping and Bcl-2 expression were associated with tumour recurrence although not with each other. Bcl-2 scoring remained significant even after adjusting for Jass grouping. This suggests that the Bcl-2 score may have additional prognostic significance. There are obviously several different properties of a tumour which will affect its behaviour and the prognosis for the patient, and it is likely that the Jass grouping and immunostaining for Bcl-2 expression are measuring different factors.

Our data raise interesting questions about tumour biology. The overexpression of Bcl-2 protein during adenomatous growth suggests that selection of Bcl-2 mediated inhibition of apoptosis is an early event in the development of colorectal tumours. It appears antithetical that, both in our study and those of others, tumours which lose this means of inhibition of apoptosis actually have a worse prognosis than tumours which retain Bcl-2 expression. As adenomatous growth and tumour invasion are two different phases of tumour progression, it may be that during the phase of tumour invasion, the apoptotic stresses are different and this necessitates selection of a different means of inhibition of apoptosis (such as p53 mutation). In this case, Bcl-2 function would become redundant and could be lost with no cost to the tumour. Alternatively, it may be that Bcl-2 function may be sacrificed for another advantage (such as loss of an oncosuppressor gene on chromosome 18). Either way, these data dovetail, to some degree, with the data showing a correlation between loss of expression of DCC protein and poor prognosis[26] as both the Bcl-2 and DCC genes occur on chromosome 18q21.

In conclusion, we have shown that high Bcl-2 expression is associated with freedom from recurrence in moderately differentiated Dukes' B colorectal carcinoma. This raises the possibility of the use of Bcl-2 expression as an adjunctive test in the clinical management of this group of tumours. Although the Bcl-2 scores obtained in this study were the combined scoring of two separate pathologists, these results need to be corroborated by other similar controlled studies in order to ascertain the true value of Bcl-2 immunostaining in colorectal cancer.

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