Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumour stage and patient survival

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Summary The *bcl-2* gene encodes for a mitochondrial membrane proto-oncoprotein, the expression of which is known to suppress programmed cell death (apoptosis). In the present study the prognostic value of bcl-2 proto-oncoprotein was immunohistochemically investigated in a series of 104 colorectal carcinomas. The bcl-2 staining patterns were semiquantitatively assessed and correlated with the pTNM stage, Dukes' classification, lymphocytic infiltration (Jass classification) and tumour grade as well as parameters not associated with prognosis (gender, age, tumour site, histological tumour type). Statistical analysis was carried out using the Kaplan–Meier method, the log-rank test, hazard ratios and their confidence intervals. Fifty-five out of 104 cases completely lacked immunohistochemical bcl-2 expression. Fewer than 5% of bcl-2-positive cells were found in 25, 5–50% in 17 and more than 50% in five cases. The extent of bcl-2 expression by tumour cells decreased significantly with respect to increasing tumour size ($P<0.05$), decreasing lymphocytic infiltration ($P<0.05$) and chance of poor clinical outcome ($P<0.05$), but not with worsening of Dukes stages. In multivariate analysis (Cox regression model) bcl-2 expression remained as an independent prognostic parameter with Dukes' classification as stratification factor ($P<0.001$). Although the biological functions of bcl-2 protein are not yet well understood, our results provide further evidence that bcl-2 oncoprotein appears to be associated with favourable clinical outcome. Thus immunohistochemical bcl-2 phenotyping of colorectal carcinoma may contribute in future to the clinical management of these patients.

Keywords: bcl-2; immunohistochemistry; colorectal cancer

The *bcl-2* gene encodes for a membrane-associated protein that is present in outer mitochondrial membrane. Additionally it was described in some parts of the endoplasmatic reticulum and nuclear envelope (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; Jacobson *et al.*, 1993). Expressed widely during embryonic development, in the adult it is confined to long-lived cells (e.g. stem cell populations, resting B lymphocytes, and peripheral neurones) (Hockenbery *et al.*, 1991). The biochemical function remains largely unknown, although bcl-2 oncoprotein is known to inhibit programmed cell death (for review see Reed, 1994). Furthermore some data support a role in cell growth control via regulation of the redox system of cells (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Richter, 1993). Recently a paradoxical inhibition of *in vitro* cell growth has been reported in several solid tumour cell lines (Pietenpol *et al.*, 1994).

The *bcl-2* gene product was shown to be over-expressed in the 14;18 translocation of human B-cell lymphoma (Tsujimoto *et al.*, 1985), Hodgkin's disease, and reactive lymph nodes (Corbally *et al.*, 1994) and was immunohistochemically demonstrated in breast carcinoma (Doglioni *et al.*, 1994; Leek *et al.*, 1994; Silvestrini *et al.*, 1994), follicular carcinoma of the thyroid (Pilotti *et al.*, 1994a,b), non-small-cell lung cancer (Pezzella *et al.*, 1993), hepatocellular carcinoma (Zhao *et al.*, 1994) and neuroblastoma (Castle *et al.*, 1993), but not yet reported in colorectal cancer. In the present study 104 colorectal carcinomas were immunohistochemically investigated with a monoclonal antibody reactive against bcl-2 oncoprotein. The study was designed to assess the extent of the immunohistochemical bcl-2 expression by tumour cells as a possible prognostic parameter in colorectal cancer with regard to patients' survival as well as histological grading and staging parameters.

Patients and methods

Tumour tissues of 104 consecutive cases of colorectal adenocarcinoma (40 rectal carcinomas, 43 carcinomas of the left, and 21 of the right colon; 54 male, 50 female patients; mean age 67.8 years, ranging from 35 to 90 years) were investigated in this study. All patients had been operated between 1984 and 1986 at the Department of Surgery I, Innsbruck University Hospital, Austria, either with curative ($n = 87$) or palliative intent ($n = 17$). None of these patients died within 30 days after surgery, were treated with adjuvant chemo- and/or radiotherapy or were members of families with familial adenomatosis coli or hereditary non-polyposis colorectal cancer.

Tumour tissues were routinely processed (formalin-fixed and paraffin-embedded) and were classified according to Dukes' classification (Dukes and Bussey, 1958), with an added D-stage for patients with distant metastases, TNM staging system (Spiessl *et al.*, 1992) and WHO grading system (Morson and Sobin, 1976). Lymphocytic infiltration at the advancing edge of the tumour was determined according to the criteria of Jass *et al.* (1986). Table I provides a detailed description of staging and grading results. The mean follow-up period of the 52 patients still alive until April 1994 was 79 months. The fate of the remaining three patients could not be ascertained as they failed to comply with the follow-up scheme.

Staining procedures

The commercially available monoclonal anti-bcl-2 antibody 124 (Dako, Copenhagen, Denmark) was applied to sections that were pretreated with the wet autoclave method (Bankfalvi *et al.*, 1994) overnight at 4°C in a humidified chamber [dilution in phosphate-buffered saline (PBS) containing 0.6% bovine serum albumin 1:300], followed by a goat anti-mouse bridging antibody (1:30 in PBS; 30 min at room temperature; Dako) and a polyclonal mouse APAAP complex (1:100 in

PBS; 60 min at room temperature; Dianova, Hamburg, Germany). The bridging antibody and the APAAP complex were applied on a semiautomatic immunostaining device ('Omnibus'; Quartett, Berlin, Germany). Subsequently the enzyme reaction was developed for 25 min at room temperature in a freshly prepared new fuchsin solution containing naphthol-bi-as-phosphate. Finally the sections were counterstained with haematoxylin and mounted in Kayser's glycerine gelatine. Omission of the primary antibody and replacement of the primary antibody by an inappropriate monoclonal antibody were used for negative control, and normal human tonsil tissues for positive control reactions.

Semiquantitative assessment of immunohistochemical staining patterns

Semiquantitative evaluation was performed twice by one of the authors (KWS) with a 2 week interval. The staining was categorised as follows: no immunoreactive cells detectable (neg), fewer than 5% bcl-2 (+), 5–50% of tumour cells (+ +), and more than 50% (+ + +) positive cells. The semiquantitative evaluation was shown to be highly reproducible since no divergent diagnoses was made in the second assessment.

Patients' follow-up and statistical analysis

All data were entered into a Macintosh IIci microcomputer and statistical analysis was carried out using the SYSTAT statistical package (Wilkinson, 1989) including the SURVIVAL supplementary module (Steinberg and Colla, 1988). Patients were followed up according to the oncological

Table I Frequency of prognostic parameters investigated in 104 colorectal adenocarcinomas

Paramater	No.	Percentage
Tumour type		
Intestinal	82	79
Mucinous	17	16
Signet ring cell	5	5
Histological grading		
Well	14	13
Moderate	65	63
Poor	25	24
Lymphocytic infiltration		
Mild	43	41
Moderate	46	44
Significant	15	15
Dukes' stage		
A	9	9
B	48	46
C	30	29
D	17	16
pT stage		
pT1	10	10
pT2	4	3
pT3	80	77
pT4	10	10
pN stage		
pN0	54	52
pN1	13	12
pN2	20	19
pN3	5	5
pNx	12	12
M stage		
M0	87	84
M1	17	16
Tumour site		
Right hemicolon	21	20
Left hemicolon	43	41
Rectum	40	39
Age		
≤ 65 years	34	33
> 65 years	70	67
Sex		
Male	54	52
Female	50	48

follow-up scheme of the Department of Surgery I, University of Innsbruck: clinical and laboratory examination (including tumour marker CEA) were performed every 3 months within the first 3 years, every 6 months in the 4th and 5th years after surgery, and once a year afterwards. Colonoscopy or barium enema and chest radiograph were performed according to an obligatory protocol twice in years 1–3 and once a year until year 5 after operation. Additionally the data concerning the date and cause of death were confirmed by the 'Österreichisches Statistiches Zentralamt', an institute of the Austrian government. The cumulative patient survival was estimated with the Kaplan–Meier method (Kaplan and Meier, 1958); for comparison of the survival curves the log-rank test was used (the Mantel–Haenszel method; Kalbfleisch and Prentice, 1980). The Cox proportional hazards linear regression model (Cox, 1972), with Dukes' stages as a stratification factor, was used to determine, in a forward stepwise procedure, which factors were associated simultaneously with survival. Estimates of relative risks and 95% confidence intervals (CI) were generated from the parameters estimates of regression coefficients and associated standard errors. Descriptive statistics for continuous measures are given as the mean with the respective standard deviation in parenthesis; discrete data frequency counts and percentages are tabulated and groups were compared using chi-square analysis with Yates' correction whenever appropriate.

Results

Immunostaining for bcl-2 protein

Fifty-five out of 104 cases (55%) investigated completely lacked immunohistochemically detectable bcl-2. Less than 5% of bcl-2-positive cells were found in 25 (24%), 5–50% in 17 (16%), and more than 50% in five cases (5%) investigated (Table II). In areas of normal colonic epithelium adjacent to the tumour, bcl-2 expression was found in some basal cells. Differentiated epithelial cells in the colonic mucosa were constantly negative. A strong bcl-2 expression was found in reactive lymphocytic cells. All negative controls constantly lacked bcl-2 staining. Figure 1a–c depicts representative immunohistochemical staining results.

Correlation of bcl-2 expression with clinicopathological parameters

The extent of bcl-2 expression by tumour cells decreased in a statistically significant way [chi-square, 19.4; degrees of freedom (DF), 9; $P = 0.02$; Table III] with increase in tumour size (pT), but not Dukes' stages (total chi-square, 15.0; DF, 9; not significant). Tumour bcl-2 staining also increased significantly with increasing lymphocytic infiltration at the advancing edge of the tumour (total chi-square, 13.2; DF, 6; $P = 0.04$) and chance of uneventful clinical outcome (total chi-square, 13.6; DF, 6; $P = 0.03$). Table IV summarises all correlations of bcl-2 expression to clinicopathological parameters available.

Univariate and multivariate long-term survival analysis

Figure 2 illustrates survival curves for all patients in the study with regard to bcl-2-negative and -positive immuno-

Table II bcl-2 expression of 104 colorectal carcinomas with regard to Dukes' classification

bcl-2 expression	Dukes A	Dukes B	Dukes C	Dukes D
neg	2	26	19	10
+	5	11	5	4
+ +	0	9	5	3
+ + +	2	2	1	0

(Chi-square, 15.0; degrees of freedom, 9; not significant). Neg, no immunoreactive cells detectable; +, less than 5% bcl-2; + +, 5–50% tumour cells; + + + >50% positive cells.
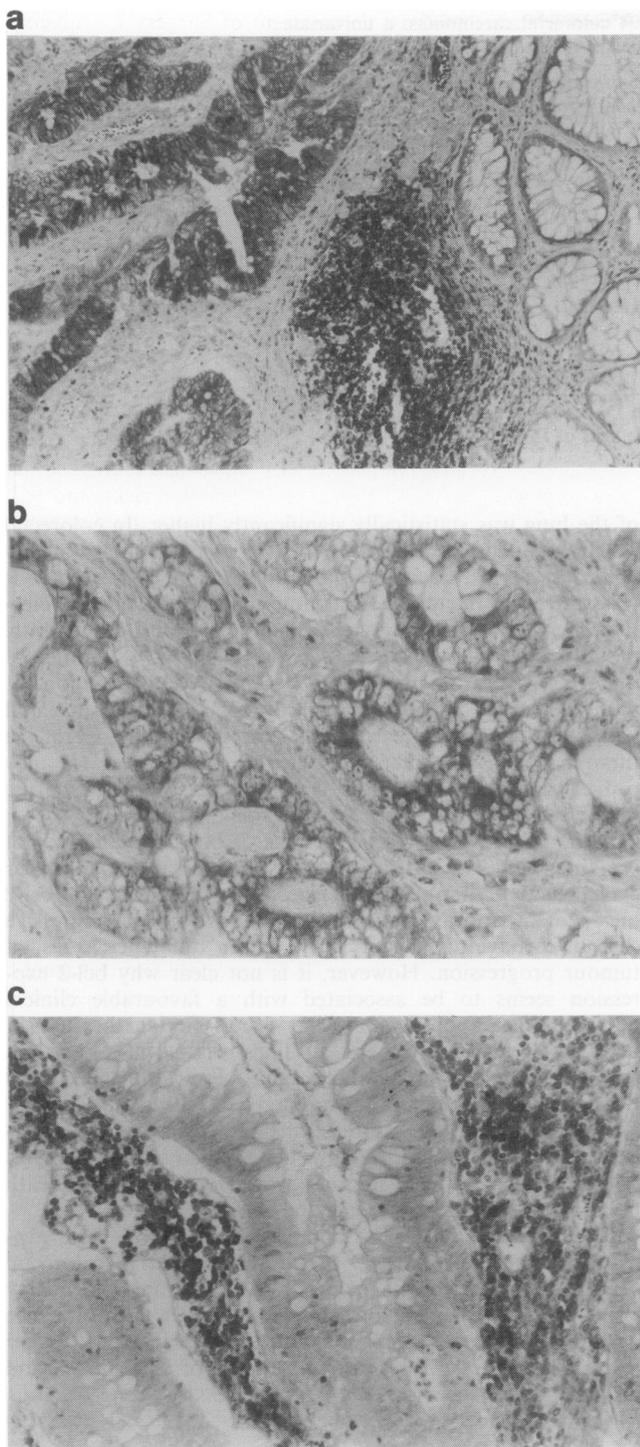
Figure 1 (a) Well differentiated to moderately differentiated colonic carcinoma with pronounced immunohistochemical bcl-2 expression. Note strong bcl-2 immunoreactivity of lymphocytes. Normal colonic mucosa situated adjacent to the tumour lacks bcl-2 (APAAP, × 100). (b) Less differentiated colonic carcinoma with moderate cytoplasmic bcl-2 expression (APAAP, × 250). (c) Well-differentiated colonic carcinoma lacks immunohistochemically detectable bcl-2, whereas bcl-2 can be demonstrated in lymphocytes (APAAP, × 250).

Table III bcl-2 expression of 104 colorectal carcinomas with regard to pT stage

bcl-2 expression	pT1(%)	pT2(%)	pT3(%)	pT4(%)
neg	2 (20)	2 (50)	44 (55)	8 (80)
+	5 (50)	0 (0)	18 (23)	2 (20)
+ +	1 (10)	1 (25)	16 (20)	0
+ + +	2 (20)	1 (25)	2 (2)	0

(Chi-square, 19.4; degrees of freedom, 9; $P = 0.02$). Neg, no immunoreactive cells detectable; +, less than 5% bcl-2; + +, 5–50% tumour cells; + + +, 75% positive cells.

Table IV Correlation between bcl-2 and various clinicopathological parameters investigated

Parameter	Chi-square	DF	P
Dukes' classification	15.0	9	NS
pT stage	19.4	9	0.02
pN stage[a]	4.7	9	NS
M stage	1.1	3	NS
Histological tumour grade	7.7	6	NS
Tumour type	5.0	6	NS
Tumour site	4.1	6	NS
Lymphocytic infiltration	13.0	6	0.04
Age ($\leqslant 65$ years $vs > 65$ years)	0.2	3	NS
Sex	6.0	3	NS

NS, not significant; DF, degrees of freedom. [a]pNx cases ($n = 13$: four Dukes A and nine Dukes D cases) excluded.
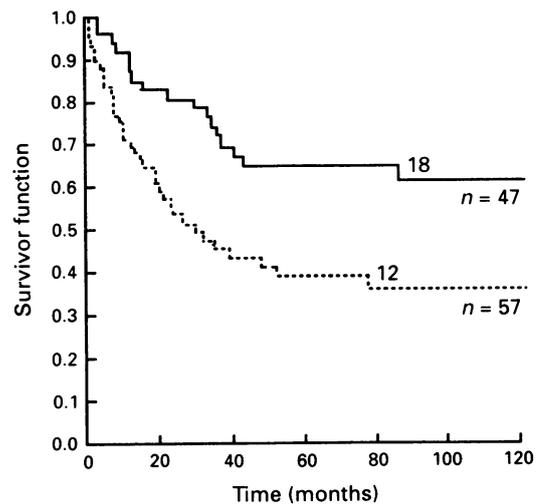

Figure 2 Kaplan–Meier survival curves of 104 colorectal carcinomas with regard to bcl-2-positive and -negative immunoreactivity. Chi-square for the log-rank test (Mantel–Haenzel method), 8.3; degrees of freedom, 1; $P = 0.004$. —— bcl-2 positive; ·········· bcl-2 negative.

reactivity. Univariate survival analysis of parameters significantly associated with survival (Dukes' stage, pT stage, pN stage, M-stage, lymphocytic infiltration, bcl-2 scores, histological tumour grade) and variables which were not associated with prognosis (sex, age, tumour site, histological tumour type) are given in Table V. Multivariate analysis by means of the Cox regression model using Dukes' classification as a stratification factor (relative hazard rates for patients with different Dukes' stages were not constant

over the follow-up intervals) revealed that bcl-2 immunoreactivity was independently and statistically highly significantly associated with survival (T-Stat, -3.1; $P = 0.001$). Additionally a marginal significance was found for the lymphocytic infiltration (Jass classification; T-Stat, -1.73; $P = 0.05$). All other parameters which were significant by univariate approach were rejected in multivariate analysis.

Discussion

Immunohistochemical phenotyping of tumours may provide important information concerning tumour behaviour. Although the known function of bcl-2 indicates a possible prognostic role for this oncoprotein, only a few investigations have compared immunohistochemically demonstrable bcl-2 protein with clinicopathological parameters. Our immunohistochemical results on tumours from patients with colorectal carcinoma revealed a statistically significant association of

Table V Prognostic factors examined in 104 colorectal carcinomas: a univariate approach to cancer-specific mortality

	Univariate χ^2 for the log-rank test	DF	p
Dukes' stage	84.8	3	0.0001
pT stage	17.3	3	0.001
pN stage[a]	30.5	4	0.0001
M stage	78.5	1	0.0001
bcl-2 expression (negative vs positive)[b]	8.3	1	0.004
Lymphocytic infiltration[b]	8.6	2	0.01
Histological tumour grade	7.6	2	0.02
Type of tumour	4.1	3	NS
Tumour site	2.4	2	NS
Age (≤ 65 years vs > 65 years)	0.0	1	NS
Sex	0.0	1	NS

[a]pNx cases ($n = 13$: four Dukes A and nine Dukes D cases) excluded. [b]Remained significant by Cox regression analysis. DF, degrees of freedom.

bcl-2 expression with favourable clinical outcome. Similar results were previously reported on non-small-cell carcinomas of the lung (Pezzella et al., 1993), breast carcinoma (Leek et al., 1994) and thyroid follicular carcinomas (Pilotti et al., 1994b). As in these tumours, the classic cause of bcl-2 overexpression, namely 14;18 translocation, has not been found in colorectal carcinoma.

Our results showed that immunohistochemical bcl-2 demonstration was significantly associated with the pT stage but not with Dukes' classification or the development of lymph node and organ metastases. bcl-2 was detectable in more than 60% of cases with uneventful clinical course, whereas the majority of cases with the development of metastases (62%) and local recurrence (>90%) completely lacked immunohistochemically detectable bcl-2 protein. These findings support the concept that bcl-2 expression is related to slower local tumour growth. Thus, the better clinical outcome of these patients may simply be attributable to the prolonged period over which these tumours remain clinically detectable in their earlier stages of progression.

Normal tissues of organs with slow cell turnover rates are known to express bcl-2 protein (Hockenbery et al., 1991; Doglioni et al., 1994; Leek et al., 1994; Pilotti et al., 1994b). In contrast to studies performed on carcinomas of the breast (60%) and carcinomas derived from thyroid follicular epithelium (approximately 80%), we found a considerably lower percentage of positive immunoreactive cases in colorectal cancer (45%). This most likely reflects biological differences between these tumour types. It is intriguing to see that, in contrast to the very advanced tumours of our series of colorectal carcinomas expressing bcl-2 (<5%), bcl-2 was detectable in more than 80% of poorly differentiated thyroid carcinomas (Pilotti et al., 1994a).

In the studies on breast and thyroid carcinomas bcl-2 expression was shown to correlate with single favourable prognostic parameters. However, in colorectal cancer a statistically significant inverse association ($P = 0.02$; Table III) between bcl-2 expression and tumour size was found. Immunoreactivity for bcl-2 decreased from 80% (pT1 tumours) to 20% (pT4 tumours) with progressive tumour size (compare Table III). Pezzella et al. (1993) found in their study that survival of patients with bcl-2-positive non-small-cell carcinomas (80 squamous-cell and 42 adenocarcinomas)

of the lung was statistically significantly higher. In colorectal cancer a similar association ($P = 0.03$) of bcl-2 immunoreactivity and clinical course of patients was found. Moreover it was proven in our study, that immunohistochemically detectable bcl-2 expression predicts patient survival independently in uni- and multivariate analysis ($P = 0.001$). Although of minor statistical significance ($P = 0.04$), a correlation of immunohistochemically demonstrated bcl-2 expression and lymphocytic infiltration patterns at the advancing edge of the tumour (Jass et al., 1986) was shown. Further research may prove whether bcl-2 expression may be associated with the biological background of lymphocytic infiltration.

Although the biological functions of bcl-2 protein are not yet fully understood, the present findings provide further evidence that immunohistochemically demonstrated bcl-2 oncoprotein appears to be associated with less aggressive tumour behaviour and/or may reflect different stages of tumour progression. However, it is not clear why bcl-2 expression seems to be associated with a favourable clinical outcome. Pietenpol et al. (1994) found that expression of bcl-2 in several solid tumour cell lines resulted in a paradoxical growth inhibition similar to that seen with p53. It has been suggested that bcl-2-promoted cell survival in slowly growing tumours may decrease the rate of acquiring complementary defects. Additionally bcl-2 oncoprotein regulates cell growth not only by inhibition of programmed cell death but also via the redox system of cells.

In summary we propose immunohistochemical bcl-2 expression to be an additional prognostic marker in colorectal carcinoma. Immunohistochemically demonstrated bcl-2 may even be an independent prognostic parameter with influence on post-operative therapeutic strategies. However, further research is necessary to elucidate the role of bcl-2 in cell and tumour growth control.

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