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European Journal of Cancer 36 (2000) 348–356

European Journal of Cancer

www.elsevier.com/locate/ejconline

p53 and Bcl-2 as significant predictors of recurrence and survival in rectal cancer

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Received 15 April 1999; received in revised form 9 September 1999; accepted 13 October 1999

Abstract

The aim of this study was to evaluate the prognostic value of p53 nuclear accumulation and Bcl-2 expression after curative surgery for rectal cancer. Immunohistochemistry was performed using monoclonal antibodies (MAb) (DO-1 for p53; anti-human Bcl-2 MAb, clone 124, for Bcl-2) on formalin-fixed, paraffin-embedded tissues of 160 rectal carcinomas (UICC stages I–III), and results were compared with data from the prospective registry of rectal cancer by univariate and multivariate logistic regression model focusing specifically on recurrence. Survival was calculated by the Kaplan–Meier method and proportional hazards model. p53 nuclear accumulation was documented in 39% ($n=63$) of tumours and was associated with a higher incidence of tumour progression (local or distant recurrence) and poorer disease-free survival ($P<0.0001$). Bcl-2 expression was detected in 29% ($n=47$), and was associated with longer disease-free survival and lower incidence of recurrence ($P<0.0086$). Multivariate logistic regression analysis demonstrated that gender ($P=0.0136$), UICC stage ($P=0.0002$), p53 expression ($P=0.0002$) and Bcl-2 expression ($P=0.0243$) were independent factors predictive of recurrence. The proportional hazards model identified p53 ($P=0.0009$), UICC stage ($P=0.0480$), gender ($P=0.0049$), but not Bcl-2 ($P=0.1503$), as independently related to disease-free survival. Looking at the p53/Bcl-2 subgroups, the poorest prognosis was observed in the p53+/Bcl-2− subgroup, whereas patients whose tumours were p53−/Bcl-2+ had the best prognosis ($P<0.0001$). Immunohistochemical assessment of both p53 and Bcl-2 status may be valuable in predicting recurrence and survival after curative surgery for rectal cancer. Therefore, they play a role as prognostic factors in rectal cancer. p53 is a stronger predictor of prognosis than Bcl-2.

Keywords: Rectal cancer; Immunohistochemistry; p53; Bcl-2; Prognosis; Recurrence

1. Introduction

Colorectal cancer is a common cause of morbidity and mortality with approximately 300 000 new cases and 200 000 deaths in Europe and the USA each year [1]. In approximately 40% of cases, the cancer originates from the rectum. Both the product of the tumour suppressor gene *TP53* and the Bcl-2 protein play a central role in the regulation of cell proliferation and apoptosis, and alterations in these genes are related to oncogenesis and tumour progression [2–5]. In response to DNA damage, p53 either arrests cells in the G_1 phase of the cell cycle or if damage is irreparable commits cells to the apoptotic cell suicide pathway [6]. Furthermore, p53 is an inhibitor of Bcl-2 expression, and promotes the synthesis of bax, which induces programmed cell death by complexing with Bcl-2. Mutations in the *TP53* gene are the most common genetic lesions occurring in human cancer, and have been implicated in the development of colorectal carcinoma [7]. Loss of the wild-type phenotype of *TP53* often results in the nuclear accumulation of mutated proteins due to their increased half-lives which can be detected by routine immunohistochemistry [8,9].

Bcl-2 is an intracellular membrane protein capable of inhibiting programmed cell death [10]. The *Bcl-2* gene is overexpressed in follicular B-cell non-Hodgkin's lymphomas resulting from a t(14;18) translocation; however, overexpression of Bcl-2 has also been detected in human epithelial tumours without translocation [11]. Several studies have examined immunohistochemically

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0959-8049/00/$ - see front matter
PII: S0959-8049(99)00271-3

the prognostic value of p53 and Bcl-2 in colorectal cancer, but contradictory results have been reported: expression of Bcl-2 in colorectal cancer has been demonstrated as being a favourable prognostic factor [12–14], however, other authors did not find any prognostic significance [15,16]. Currently available data on the prognostic value of p53 nuclear accumulation in colorectal cancer are controversial due to discrepancies in the methods used [17,18], and the correlation of *TP53* abnormalities to patient prognosis still remains unclear [13,16]. However, to our knowledge, there is no study currently available solely for rectal cancer in which p53 and Bcl-2 expression are immunohistochemically assessed and related to prognosis. Therefore, it was our aim to analyse immunohistochemically Bcl-2 expression and p53 nuclear accumulation in a series of 160 rectal carcinomas, and to determine the prognostic value of detecting one or both oncoproteins.

2. Patients and methods

2.1. Patients, surgery and follow-up

Within a 5-year period (January 1993 and December 1997) 172 patients underwent curative surgery for rectal cancer (UICC stages I–III) at the Department of Surgery, Medical University of Luebeck. A carcinoma was considered a primary rectal carcinoma if it was located in the lower third (0–6 cm from the anal verge), middle third (7–12 cm), and upper third of the rectum or rectosigmoid junction (above 12 cm). For the current study, patients with familial adenomatous polyposis ($n=1$), inflammatory bowel disease ($n=2$), synchronous colon cancer ($n=4$), and patients who died within 30 days after surgery ($n=5$) were excluded from analysis, leaving 160 patients with rectal cancer (mean age 66.7; range: 31–92 years; 79 males and 81 females; tumour stages: UICC I: $n=64$, UICC II: $n=33$, UICC III: $n=58$, and five low-risk pT1 rectal carcinomas in which local excision was performed) to be enrolled in this analysis. Clinical, operative, histopathological and follow-up data have been recorded prospectively in a computerised registry database including patient age, gender, tumour site, tumour stage according to UICC stage [19], histological differentiation, gross morphology, tumour size, local invasion, nodal status, type of surgery and adjuvant therapy and follow-up information. Oncological resection was performed in a standardised technique [20]. Following R0 resection of rectal carcinomas, patients with UICC stages II and III (pT3/4 or pN+) underwent adjuvant therapy including postoperative radiotherapy with concurrent chemotherapy (5-fluorouracil, folinic acid). All patients were followed up prospectively. Follow-up included patient history, physical examination, laboratory investigations, abdominal ultrasonography, chest X-ray and endoscopy (sigmoidoscopy after 6 months, total colonoscopy after 1 year). Assessments were performed initially every 3 months, then semi-annually during the period of 2–5 years postoperatively; thereafter, follow-up was conducted either solely based on symptoms, or annually. Follow-up focused specifically on recurrence (local and distant), disease-free survival, overall survival and cancer-related death, and data collected were entered prospectively into the registry database. Mean follow-up was 38 (range: 12–72) months.

2.2. Tissue samples

One hundred and sixty formalin-fixed, paraffin-embedded tissue samples of rectal cancer (kindly provided by A.C. Feller, Institute of Pathology, Medical University of Luebeck, Germany) were cut into sections of 4 μm, mounted on Superfrost™ slides (Menzel, Braunschweig, Germany) and dried overnight at 56°C. Representative sections were stained with haematoxylin–eosin prior to immunostaining to ensure that the slides contained tumour tissue as the regions of interest.

2.3. Immunohistochemistry: p53

The slides were dewaxed in xylene, rehydrated in graded acetone and pretreated for antigen retrieval in 10 mM citrate buffer (pH 6.0) in a microwave oven twice for 10 min, each at 600 W. After quenching endogenous peroxidase activity with 0.5% hydrogen peroxide for 30 min, non-specific binding of the antibody was blocked by 20% normal goat serum for 20 min at 25°C. The specimens were incubated with the p53 monoclonal antibody DO-1 (Oncogene Science, Uniondale, NY, USA) in a dilution of 1:100 overnight at 4°C. The monoclonal antibody (MAb) DO-1 recognises an epitope of both wild-type and mutant p53 protein. For visualisation of p53 nuclear accumulation, the avidin–biotin complex (ABC) method by the Strept-ABC-Kit™ (Dako Diagnostika, Hamburg, Germany) was applied. For subsequent staining, AEC (3-amino-9-ethylcarbazole) was used as chromogen, and the nuclei were counterstained with haematoxylin. For a positive control, colonic adenocarcinoma known to express p53 was used, and omitting the primary antibody served as negative control.

2.4. Immunohistochemistry: Bcl-2

The sections were dewaxed in xylene, rehydrated in graded alcohols, pretreated for antigen retrieval in 10 mM citrate buffer (pH 6.0) in a microwave oven for 15 min at 750 W, and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide. Slides were incubated with a 1:20 dilution of the primary mouse

antihuman Bcl-2 MAb (clone 124) (Dako Diagnostika) for 30 min at room temperature. The MAb Bcl-2 reacts specifically with the Bcl-2 oncoprotein in the cytoplasm or on the perinuclear membrane. For subsequent staining, the EnVision+™ two-step visualisation technique (Dako Diagnostika) was applied. AEC (3-amino-9-ethylcarbazole) was used as chromogen, and slides were counterstained with haematoxylin. For Bcl-2 staining, human tonsil served as positive control showing strong staining in the mantle zone. Infiltrating lymphocytes were used as internal positive control in every section of tumour. In negative controls, the primary antibody was omitted.

2.5. Assessment of p53 nuclear accumulation and Bcl-2 expression

p53 expression (nuclear staining) and Bcl-2 expression (cytoplasmic or perinuclear staining) were evaluated by counting 1000 cells/section in five randomly high-power fields (400×) of tumour, and the percentages of positive cells (red staining) were determined by light microscopy.

A tumour was considered as p53-positive if more than 10% of tumour cells showed positive nuclear immunoreactivity. p53 nuclear staining was graded semi-quantitatively in categories 0–3 where 0 = no staining; 1 = 10% or less; 2 = more than 10% but less than 50%; 3 = more than 50%.

A tumour was considered as Bcl-2-positive if more than 10% of tumour cells showed positive immunoreactivity, and Bcl-2 staining was scored in the same manner (0 = no staining; 1 = 10% or less; 2 = more than 10% but less than 50%; 3 = more than 50%).

All slides were examined and scored independently by two investigators in blinded fashion without knowledge of clinical or histopathological data. The results of the two reviewers achieved correspondence in 95%; however, tumours in which results diverged initially were re-evaluated in order to reach consensus.

2.6. Statistical analysis

Statistical analysis was performed to determine whether clinical variables, histopathological data or p53 and Bcl-2 expression, could be used to predict patients who were at an increased risk of recurrence. The correlation between various clinicopathological variables and p53 or Bcl-2 expression was evaluated by univariate analysis using chi-squared test and Student's *t*-test to assess categorical and continuous data, respectively. For multivariate analysis, the logistic regression analysis was applied to determine independent factors predictive of recurrence by using the NCSS™ software package (NCSS, Kaysville, UT, USA). Survival curves were calculated by Kaplan–Meier estimation [21] using the SPSS™ software package (SPSS, Chicago, IL, USA), with log-rank comparisons of survival curves. Deaths due to unknown causes or related to causes other than rectal cancer were treated as censored observations at the time of death. Multivariate survival analysis was performed with the proportional hazards model by using the NCSS™ software package (NCSS). Statistical significance was assessed at the 5% level ($P < 0.05$).

3. Results

3.1. p53 nuclear accumulation and Bcl-2 expression

Of 160 rectal carcinomas, p53 nuclear accumulation was documented in 39% ($n = 63$), and Bcl-2 expression in 29% ($n = 47$) of the tumours (Fig. 1a,b). There was no significant correlation between p53 status with clinicopathological variables, such as age, gender, tumour site, UICC stage, gross morphology, histological differentation, tumour size, local invasion (pT category) or nodal status. However, there was a significant association

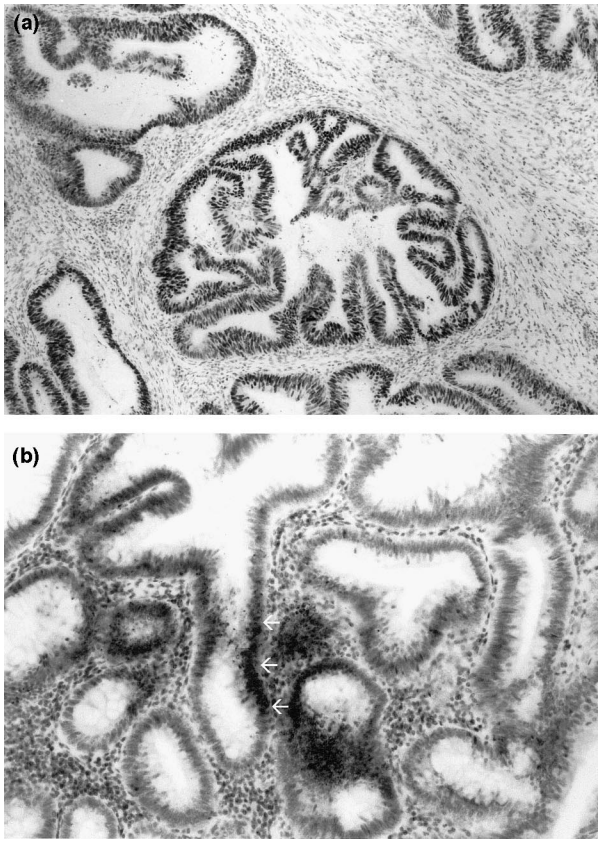

Fig. 1. Immunostaining for p53 nuclear accumulation: (a) and Bcl-2 expression in rectal cancer; (b) for p53, strong staining of the nuclei were observed (magnification 100×). The staining observed is p53-specific and not due to counterstaining as observed on colour slides (data not shown). In contrast, for Bcl-2, heterogeneous perinuclear and cytoplasmic staining was documented (arrowed) (magnification ×200).

between Bcl-2 expression and tumour stage (UICC stage I versus stage II, $P=0.0412$), and tumour penetration through the rectal wall (pT1 versus pT2, $P=0.0009$; pT1 versus pT3, $P=0.0007$) (Table 1).

If Bcl-2 expression was compared between tumours that showed or did not show p53 nuclear accumulation, neither a negative nor a positive correlation, but an inverse trend between p53 and Bcl-2 expression could be noted ($P=0.0726$, data not shown). However, when patients were stratified by both p53 and Bcl-2 status, the combination 'p53−/Bcl-2+' was mostly seen in pT1 carcinomas ($P=0.0085$, data not shown).

3.2. Association with recurrence

After a mean follow-up of 38 months (range 12–72 months), tumour progression, caused by both local recurrence ($n=10$, 6%) and distant metastases ($n=22$, 14%), occurred in 32 patients (20%). Univariate analysis showed a significant association of tumour recurrence with gender ($P=0.0143$), UICC stage ($P=0.0259$), tumour penetration through the rectal wall ($P=0.0004$) and lymph node status ($P=0.0028$) (Table 2). Patients with rectal cancer whose tumours demonstrated p53 nuclear accumulation had a significant increased occurrence of tumour recurrence (37% in p53+ versus 9% in p53−, $P<0.0001$). Conversely, a significant decrease in tumour progression was found in patients whose tumours showed Bcl-2 expression (6% in Bcl-2+ versus 26% in Bcl-2−, $P=0.0055$). The results were similar when analysis was performed solely for distant recurrence (data not shown).

Looking at the p53/Bcl-2 subgroups, assessment of tumour recurrence showed that the lowest incidence of

Table 1
Association between characteristics of rectal cancer and immunohistochemical features

Variable	*n*	p53		*P* value[a]	Bcl-2		*P* value[a]
		Positive (%)	Negative (%)		Positive (%)	Negative (%)	
Age							
< 70 years	83	30 (36)	53 (64)	NS	26 (31)	57 (69)	NS
⩾70 years	77	33 (43)	44 (57)		21 (27)	56 (73)	
Gender							
Male	79	32 (41)	47 (59)	NS	22 (28)	57 (72)	NS
Female	81	31 (38)	50 (62)		25 (31)	56 (69)	
Tumour site							
Upper rectum	48	20 (42)	28 (58)	NS	16 (33)	32 (67)	NS
Middle rectum	49	20 (41)	29 (59)		15 (31)	34 (69)	
Lower rectum	63	23 (37)	40 (63)		16 (25)	47 (75)	
Tumour stage[b]							
UICC I	64	28 (44)	36 (56)	NS	20 (31)	44 (69)	0.0412 (II versus III)
UICC II	33	12 (36)	21 (64)		7 (21)	26 (79)	NS (all others)
UICC III	58	23 (40)	35 (60)		20 (34)	38 (66)	
Gross morphology							
Polypoid	72	25 (35)	47 (65)	NS	23 (32)	49 (68)	NS
Ulcerative	72	30 (42)	42 (58)		18 (25)	54 (75)	
Others	16	6 (38)	10 (63)		6 (38)	10 (63)	
Histological grade							
Well differentiated[c]	6	2 (33)	4 (67)	NS	4 (67)	2 (33)	NS
Moderately differentiated	133	55 (41)	78 (59)		39 (29)	94 (71)	
Poorly differentiated	21	6 (29)	15 (71)		4 (19)	17 (81)	
Tumour size							
⩽3 cm	39	13 (33)	26 (67)		9 (23)	30 (77)	
> 3 cm	121	50 (41)	71 (59)		38 (31)	83 (69)	
Tumour penetration							
pT 1	26	7 (27)	19 (73)		15 (58)	11 (42)	0.0009 (T1 versus T2)
pT 2	54	24 (44)	30 (56)		15 (28)	39 (72)	0.0007 (T1 versus T3)
pT 3	75	27 (36)	48 (64)		16 (21)	59 (79)	NS (T2 versus T3)
pT 42c	5	2 (40)	3 (60)		1 (20)	4 (80)	
Nodal status[b]							
Negative	92	37 (40)	55 (60)	NS	26 (28)	66 (72)	NS
Positive	63	24 (38)	39 (62)		19 (30)	44 (70)	

NS, statistically non significant ($P>0.05$).
[a] *P* values only shown as absolute figures if statistically significant ($P<0.05$).
[b] Patients who underwent local excision for low-risk pT1 rectal cancer not included ($n=5$).
[c] Not entered into statistical evaluation.

recurrence was related to the p53−/Bcl-2+ subgroup (recurrence rate 3% [1/32], $P < 0.0001$, chi-squared test), whereas the highest occurrence of either local or distant recurrence was documented in the p53+/Bcl-2− subgroup (recurrence rate 75% [21/28], $P < 0.0001$, chi-squared test).

If p53 nuclear accumulation was classified semi-quantitatively into four categories (0–3), there was evidence of a linear trend of increasing risk of tumour recurrence ($P = 0.0003$); conversely, if Bcl-2 expression was scored, tumour recurrence was significantly increased with lower Bcl-2 scores ($P = 0.0076$). Similar results were obtained for p53 and Bcl-2 when the analysis focused only on distant recurrence (data not shown).

3.3. Multivariate analysis of recurrence

Multivariate analysis by the logistic regression model demonstrated that UICC stage (regression coefficient (RC), 1.00; standard error of the mean (SEM), 0.27; chi-square, 13.55; $P = 0.0002$), p53 nuclear accumulation (RC, 1.82; SEM, 0.49; chi-square, 13.55; $P = 0.0002$), gender (RC, 1.26; SEM, 0.51; chi-square, 6.09; $P = 0.0136$)

Table 2
Clinicopathological variables and immunohistochemical features in relation to tumour recurrence (local and distant recurrence)

Variable	*n*	Cases without recurrence ($n = 128$)	Cases with recurrence ($n = 32$) (%)	*P* value[a]
Age				
< 70 years	83	64	19 (23)	NS
⩾70 years	77	64	13 (17)	
Gender				
Male	79	57	22 (28)	0.0143
Female	81	71	10 (12)	
Tumour site				
Upper rectum	48	40	8 (17)	NS
Middle rectum	49	38	11 (22)	
Lower rectum	63	50	13 (21)	
Tumour stage[b]				
UICC I	64	59	5 (8)	0.0259
UICC II	33	26	7 (21)	
UICC III	58	38	20 (34)	
Gross morphology				
Polypoid	72	62	10 (14)	NS
Ulcerative	72	54	18 (25)	
Others	16	12	4 (25)	
Histological grade				
Well differentiated[c]	6	4	2 (33)	NS
Moderately differentiated	133	107	26 (20)	
Poorly differentiated	21	17	4 (19)	
Tumour size				
⩽3 cm	39	34	5 (13)	NS
> 3 cm	121	94	27 (22)	
Tumour penetration				
pT 1	26	25	1 (4)	0.0004
pT 2	54	49	5 (9)	
pT 3	75	51	24 (32)	
pT 4[c]	5	3	2 (40)	
Nodal status[b]				
Negative	92	80	12 (13)	0.0028
Positive	63	43	20 (32)	
p53 status				
Negative	97	88	9 (9)	< 0.0001
Positive	63	40	23 (37)	
Bcl-2 status				
Negative	113	84	29 (26)	0.0055
Positive	47	44	3 (6)	

NS, statistically non significant ($P > 0.05$).

[a] *P* values (chi-squared test) only shown as absolute figures if statistically significant ($P < 0.05$).

[b] Patients who underwent local excision for low-risk pT1 rectal cancer not included ($n = 5$).

[c] Not entered into statistical evaluation.

and Bcl-2 expression (RC, −1.60; SEM, 0.71; chi-square, 5.07; $P=0.0243$) were significant independent variables of tumour recurrence.

3.4. Survival analysis

Patients with male gender ($P=0.0399$), advanced tumour stage ($P=0.0208$) also reflected by the tumour penetration through the rectal wall ($P=0.0001$), lymph node metastases ($P=0.0082$), positive p53 nuclear accumulation ($P=0.0001$) and negative Bcl-2 status ($P=0.0086$) showed a poorer survival probability using univariate Kaplan–Meier survival estimation with log-rank comparisons (Table 3). For p53, 5-year DFS was 86% in patients with p53-negative tumours, whereas it was decreased to 49% in patients with p53-positive carcinomas (Fig. 2a); for Bcl-2, 5-year DFS was 93% in patients whose tumours expressed Bcl-2, but only 63% in patients whose tumours did not express Bcl-2 (Fig. 2b). If p53 and Bcl-2 were stratified into the subgroups, patients with p53+/Bcl-2− status had the poorest survival prognosis with a 5-year DFS of only 42%, whereas patients with p53−/Bcl-2+ status had the best prognosis with a 5-year DFS of 97% (Fig. 2c). Corresponding results were achieved for overall survival (data not shown).

Table 3
Clinicopathological variables and immunohistochemical features in relation to survival

Variable	*n*	3-Year DFS %	5-Year DFS %	*P* value[a]
Age				
< 70 years	83	83	66	NS
⩾ 70 years	77	79	76	
Gender				
Male	79	79	56	0.0399
Female	81	82	82	
Tumour site				
Upper rectum	48	82	65	NS
Middle rectum	49	81	67	
Lower rectum	63	81	75	
Tumour stage[b]				
UICC I	64	94	90	0.0208
UICC II	33	80	71	(0.0163, I versus II)
UICC III	58	74	67	(0.0089, I versus III)
Gross morphology				
Polypoid	72	89	79	NS
Ulcerative	72	75	59	
Others	16	72	72	
Histological grade				
Well differentiated[c]	6	97	97	NS
Moderately differentiated	133	82	70	
Poorly differentiated	21	76	76	
Tumour size				
⩽ 3 cm	39	87	81	NS
> 3 cm	121	79	66	
Tumour penetration				
pT 1	26	96	96	0.0001
pT 2	54	94	86	(0.0033, T1 versus T3)
pT 3	75	66	49	(0.0010, T2 versus T3)
pT 4[c]	5	–	–	
Nodal status[b]				
Negative	92	89	76	0.0082
Positive	63	68	61	
p53 status				
Negative	97	91	86	0.0001
Positive	63	66	49	
Bcl-2 status				
Negative	113	76	63	0.0086
Positive	47	93	93	

NS, statistically non significant ($P>0.05$); DFS, disease-free survival rate.

[a] *P* values (log-rank test of 5-year DFS) only shown as absolute figures if statistically significant ($P<0.05$).

[b] Patients who underwent local excision for low-risk pT1 rectal cancer not included ($n=5$).

[c] Not entered into statistical evaluation.

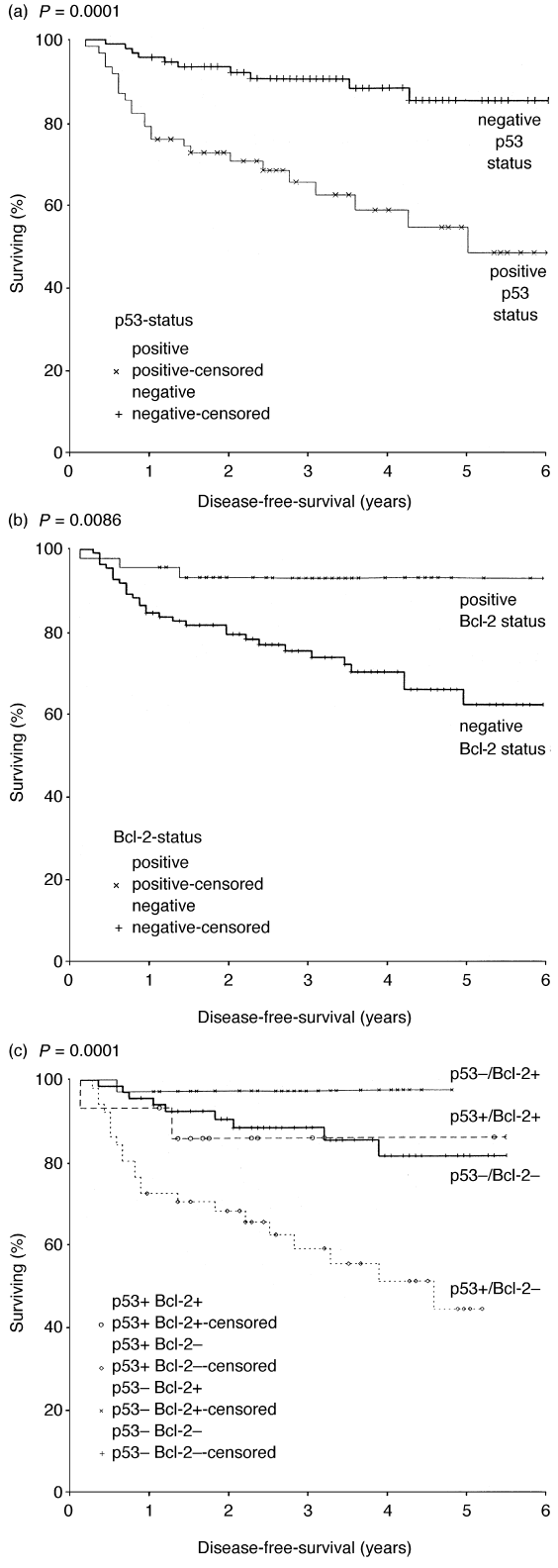

Fig. 2. Disease-free survival rates (DFS) according to: (a) p53 nuclear accumulation; (b) Bcl-2 expression; and (c) combined status of p53/Bcl-2; statistical differences of univariate Kaplan–Meier curves were calculated by log-rank comparisons; for (c) overall log-rank test for all four curves was $P=0.0001$ ($P=0.0003$ for p53−/Bcl-2− versus p53+/Bcl-2−; $P=0.0002$ for p53−/Bcl-2+ versus p53+/Bcl-2−; $P>0.05$ for others).

3.5. Multivariate survival analysis

Variables entered in the Cox proportional hazards model were p53 status, Bcl-2 status, UICC stage and gender. The multivariate proportional hazards model identified p53 nuclear accumulation (hazard ratio $=4.42$, $P=0.0009$), UICC stage (hazard ratio $=1.55$, $P=0.0480$), and gender (hazard ratio $=3.72$, $P=0.0049$) as significant independent variables related to DFS after curative surgery for rectal cancer. However, Bcl-2 expression was not an independent predictor of DFS (hazard ratio $=0.41$, $P=0.1503$).

4. Discussion

The identification of parameters that reflect biological behaviour of individual cancer tissues correlating with tumour aggressiveness is a key determinant of prognosis and a fundamental issue for the improvement of cancer therapy. Despite recent progress in defining the molecular mechanisms of cancer development and tumour progression, only few individual biomarkers providing prognostic information have been identified. However, the limited value of a single molecular marker can evidently be seen since tumours frequently express multiple proto-oncogenes or tumour suppressor genes, each of which may contribute to tumour progression. Therefore, it was the purpose of this study to assess the prognostic value of p53 and Bcl-2 expression, separately and in combination, in a group of 160 rectal carcinomas. Both the proto-oncogene *Bcl-2* and the tumour suppressor gene *TP53* are involved in the regulation of apoptosis, and although many research studies on colorectal cancer have focused specifically on both variables in relation to prognosis the results remain conflicting [12,13,16].

Technical differences are seen in varying types of fixation, types of antibodies, methods of antigen retrieval, different scoring and cut-off values [16,17]. Therefore, our results detecting p53 nuclear accumulation in 39% of rectal carcinomas may be a low percentage in comparison with p53 immunopositivity in colorectal cancer trials, although many studies have shown similar values [22,23]. The current study, and the majority of previous studies, used antigen retrieval for adequate detection of Bcl-2 expression in colorectal cancer because Bcl-2 expression is commonly low and therefore difficult to detect using immunohistochemistry, reflecting a possible downregulation of the protein in invasive carcinomas [24–26]. The incidence of Bcl-2 positive tumours in this study was similar to that noted in other studies [12,13]. According to previous studies [15,16], strong Bcl-2 immunopositivity (category 3) was rare, and more than half of the tumours showed no staining (except infiltrating lymphocytes used as internal positive control).

In our study, we identified correlations between Bcl-2 expression and tumour stage (only significant for UICC stage II versus III), and extent of rectal wall invasion as reflected by the pT category (only significant for pT1 versus pT2, and pT1 versus pT3, respectively). Previously reported associations included histological differentiation [22,24,25], lymphocyte infiltration and tumour size [15], tumour stage, lymph node invasion, and distant metastases [13]. However, other studies did not detect a significant correlation between Bcl-2 expression and any of the clinicopathological variables [16,26]. We observed this phenomenon in terms of p53 expression. From the published data focusing on the association of p53 with clinicopathological parameters of tumours, the results are far from equivocal on this subject [13,16,26].

Reasons for recurrence after curative resection for colorectal carcinoma are not completely clear. Several theories have been put forward including amongst others microscopic deposits in lymphatics, inadequate distant and lateral resection margins, exfoliated tumour cells at time of surgery, presence of malignant cells at the anastomosis, and, finally, tumour aggressiveness related to biological behaviour. It is known that reported recurrence after resection for rectal carcinoma is commonly higher than after colon carcinoma [20,27–29], and differences in prognosis have also been reported between high and low rectal carcinomas [30,31]. Risk factors that have previously been associated with increased recurrence rates include amongst others patient age, gender, tumour stage, site of lesion (colon versus rectum), infiltration of adjacent organs, histology and histopathological criteria, tumour size, lymph-node involvement, radial resection margins [27–29,32,33]. In rectal cancer, in particular, the impact of surgery and adequate lymph-node dissection related to the risk of local recurrence has been highlighted [34,35].

Several studies have evaluated the prognostic significance of both p53 and Bcl-2 on recurrence and survival in colorectal cancer, but, to our knowledge, not specifically focusing on rectal cancer. However, for colorectal carcinoma, the results on the prognostic significance of both p53 and Bcl-2 are controversial: several studies have not shown a prognostic significance [15,16]; whilst other studies have identified p53 and/or Bcl-2 as significant predictors of prognosis [12–14,22,26,36,37].

In our study, univariate analysis and the multivariate logistic regression model identified both p53 nuclear accumulation and Bcl-2 expression as significant independent predictors of recurrence following curative surgery for rectal cancer, and p53 nuclear accumulation was as strong a predictor of recurrence as UICC stage. Concerning disease-free survival, p53 nuclear accumulation and Bcl-2 expression served as prognostic factors. However, only p53 was identified as an independent predictor of disease-free survival by the multivariate proportional hazards model, whereas multivariate analysis did not identify Bcl-2 expression to be independently related to survival.

Following the results of Popescu and colleagues [38], Bcl-2 mediated inhibition of apoptosis is not a pathway by which advanced colorectal carcinoma cells avoid programmed cell death. Nevertheless, immunohistochemical evidence indicates that it may be an early step by which dysplastic cells of the colonic mucosa can accumulate genetic alterations and escape apoptosis, leading to an establishment of colorectal cancer. The low expression of Bcl-2 in colorectal cancer may represent allelic loss of the 18q chromosome, whereas the reciprocity of Bcl-2 related to p53, may be caused by a downregulation of Bcl-2 by regulators which play a later role in molecular progression to advanced cancer and to which Bcl-2 may delegate its apoptotic-inhibiting function [38]. These findings may explain our data in which low levels of Bcl-2 were associated with a poorer prognosis than Bcl-2 positive tumours. Looking at the p53/Bcl-2 subgroups in our study, patients whose tumours were p53−/Bcl-2+ had the best prognosis and least pT category, implying that these tumours form an entity with a less aggressive neoplastic transformation pathway, possibly at early stages of development.

The current immunohistochemical study demonstrates that p53 nuclear accumulation and Bcl-2 expression are significant predictors of prognosis after curative surgery for rectal cancer. In conclusion, knowledge of co-expression of apoptosis-regulating oncoproteins such as Bcl-2 and tumour-suppressor genes such as *TP53* in rectal cancer can contribute in the identification of patients with an increased risk of recurrence, and to predict prognosis. However, despite many promising studies, the potential of introducing routine p53 and Bcl-2 immunohistochemistry as a diagnostic tool into the clinical practice of rectal cancer management has still to be realised as long as the problem of reproducibility remains unsolved.

Acknowledgements

We are grateful to Professor Dr A.C. Feller, Director of the Institute of Pathology, Medical University of Luebeck, for providing tissue blocks, and we thank Mrs S. Gretsch, Institute of Pathology, for organisation. We also thank Mrs G. Grosser-Pape and Mrs A. Aumueller for technical assistance in the Surgical Research Laboratory. We also thank Mrs C. Killaitis, Department of Surgery, for processing the computerised registry database of rectal cancer. Furthermore, we are grateful to Dr H. Paul, Institute of Statistics and Mathematic Economy Research, University of Augsburg, Germany, for statistical evaluation of the data and independent review of the statistical approach.

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