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Original Paper

bcl-2 Expression is Reciprocal to *p53* and *c-myc* Expression in Metastatic Human Colorectal Cancer

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Apoptosis (programmed cell death) inhibition may be an important mechanism by which gastrointestinal mucosal cells containing damaged DNA evade normal clearance mechanisms and grow to become invasive tumours. Since *bcl-2* is an apoptosis inhibitor, *bcl-2* mRNA expression was measured in 21 metastases of colorectal cancer using reverse transcription–polymerase chain reaction analysis. The mean *bcl-2* mRNA expression (0.45 U, $P < 0.0001$) was lower than that of normal mucosal controls (= 1 U). *p53* expression was inversely correlated with *bcl-2* expression ($P = 0.021$) in 19 evaluable samples, and in tumours where *p53* expression was over twice that of normal colonic mucosal values, *bcl-2* mRNA was significantly decreased (mean 0.30, $P = 0.0052$). *c-myc* was also inversely correlated with *bcl-2* expression ($P = 0.025$). Decreased *bcl-2* expression in metastatic colorectal cancer may be partly due to allelic loss, given the proximity of *bcl-2* to the frequently deleted *DCC* gene on chromosome 18q. However, the inverse correlation to *p53*/*c-myc* suggests an active downregulation of *bcl-2*, possibly following delegation of its apoptosis inhibiting role to other genes.

Key words: *bcl-2*, *c-myc*, colorectal cancer, metastasis, mRNA, *p53*
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INTRODUCTION

In colorectal cancer, the accumulation of multiple genetic alterations in oncogenes and tumour suppressor genes results in impaired cellular growth control mechanisms causing tumour development and metastasis [1]. The most common genetic changes associated with advanced colorectal cancer are 'loss of function' mutations or deletions at *p53*, *ras*, *DCC*, *APC*/*FAP* loci and *c-myc* overexpression. Mechanisms that influence clearance of cells containing DNA damage may also be essential in preventing the establishment of pathological clones leading to tumour development, especially in tissues with high mitotic activity, such as normal gastrointestinal mucosa.

Apoptosis (programmed cell death) has been shown to be an important process by which somatically mutated cells are eliminated. Several genes are known to regulate apoptosis. The *bcl-2* proto-oncogene is an apoptosis inhibitor originally described in association with the t(14;18)(q32;q21) translocation in follicular B cell lymphoma, which places the *bcl-2* gene under the stimulatory control of the IgH promoter-enhancer at 14q32, resulting in increased *bcl-2* mRNA and protein [2] and inhibition of apoptosis. *bcl-2* expression and immunoreactivity has also been demonstrated in non-haemopoietic long lived cells and complex epithelia [3], suggesting a general role for *bcl-2* in regulating apoptosis. However, its part in the development or progression of epithelial malignancies is not yet understood. bcl-2 oncoprotein has been described in normal colonic mucosa [4–8], where it is restricted to the epithelial regenerative compartment, the intestinal crypt bases. Dysplastic colonic epithelium and normal epithelium immediately adjacent to adenocarcinomas have shown increased bcl-2 staining both in intensity and the number of bcl-2 positive cells [5, 7–9]. Adenomas have been found to express more bcl-2 than mucosal controls

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[4, 5, 7, 8], as have carcinomas in some reports [4, 5], but in other studies, adenocarcinomas displayed decreased bcl-2 immunoreactivity [7–9]. The importance of *bcl-2* in colonic tumorigenesis and the interactions of *bcl-2* with other genes involved in colorectal tumour growth and metastasis are, however, not yet understood. This is partly due to the fact that all available data on the relationship between *bcl-2* and *p53* in colorectal cancer is immunohistochemical. However, p53 immunohistopositivity gives no information on the mutational state of the *p53* gene [10] and recent evidence [11] suggests that *p53* mRNA and protein accumulation can occur in cells carrying the wild-type *p53* gene as well as in *p53* mutants. Determination of *p53* mutational status, mRNA expression and protein accumulation is important in order to allow comment on *p53* function, which is crucial in cell cycle checkpoint control and the maintenance of genome integrity by the initiation of apoptosis [12]. The cellular proto-oncogene *c-myc* is similarly involved in the regulation of cell proliferation, transformation and apoptosis and has been shown to interact with *bcl-2* in apoptosis [13].

In this study, we determined *bcl-2* mRNA expression in normal colonic mucosa and 21 colorectal tumour metastases, which we correlated to our findings of *p53* expression and mutation status, expression and amplification of *c-myc*, as well as mutations in the *ki-ras* oncogene, in the same collection of colorectal cancer specimens [14–16].

MATERIALS AND METHODS

Tumour specimens and preparation for further analysis

Material from 21 liver metastases of patients with colorectal cancer referred for metastasectomy to a tertiary referral centre was included in the study. To ensure a high tumour cell content (of at least 70%) microslides from cryostat sections were analysed and pooled as previously described [14, 16]. Normal colonic mucosa was obtained from individuals undergoing colonoscopy. Total RNA was purified from tissue homogenate (RNAzol-kit, Cinna Biotech Laboratories, Houston, Texas, U.S.A.) and reverse transcribed into cDNA using M-MLV-Reverse Transcriptase (Gibco/BRL, Bethesda, Maryland, U.S.A.) and random hexamer priming according to standard procedures.

Polymerase chain reaction (PCR) amplification, p53 *sequencing and immunohistochemistry*

Differential PCR was used to co-amplify the *p53* and *c-myc* target cDNA together with the beta-2 microglobulin (*β2-M*) reference gene in the same vessel, as described previously [14, 15]. This differential PCR technique has been described and used extensively by our group and shows a standard deviation of 20% in our hands [14–17]. This technique is thus as reproducible as other methods of mRNA quantification, such as Northern blots, which are also semiquantitative and also rely on ratios between the target gene and a reporter gene like *β-actin*. Reverse transcription–PCR (RT–PCR) is increasingly accepted as a quantification method for small amounts of RNA [11].

The sequencing of the entire coding region of *p53* and the immunohistochemistry has been described previously [14]. Briefly, following amplification, PCR products were purified using the Gene Clean procedure (Bio 101, La Jolla, California, U.S.A.) and single strands were generated by attachment to paramagnetic beads using biotin–streptavidin binding and alkaline denaturation, as recommended by the manufacturer (Dynabeads M-280, Dynal, Hamburg, Germany). Solid-phase sequencing was carried out based on the Sanger-dideoxy-method using a commercially available kit (Sequenase 2.0, United States Biochemicals). Immunohistochemical detection of p53 protein was performed with the mouse anti-p53 monoclonal antibody PAb1801 (Dianova, Hamburg, Germany) and for the visualisation of antibody binding sites, the APAAP (alkaline phosphatose anti-alkaline phosphatose) technique was used [14].

bcl-2 gene mRNA expression was measured by simultaneous cDNA amplification of the target gene (*bcl-2*) and a reference gene (*β actin*) in two different tubes. Primers used in the PCR reactions were synthesised according to published sequences [18, 19]. The 100 µl PCR mixture containing 10 µl 10×PCR buffer (0.5 M KCl, 0.1 M Tris–HCl, 15 mM $MgCl_2$, 0.01% gelatine, pH 8.3), 8 µl 10 mM deoxynucleotide mixture, 2 µl of the corresponding 10 mM oligonucleotides, cDNA and 0.5 µl Taq polymerase was amplified by a preliminary cycle of 90°C denaturation for 4 min, 55°C annealing

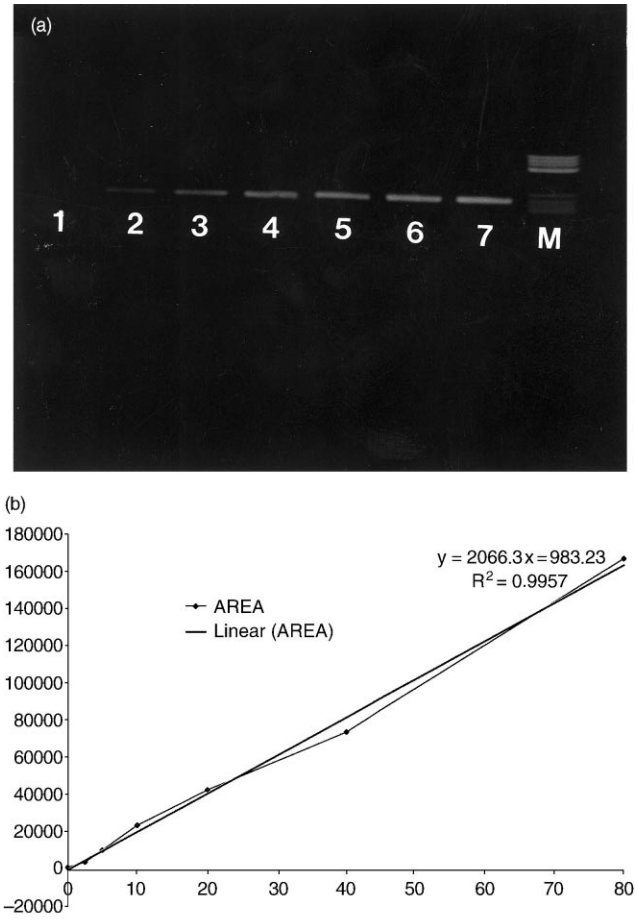

Figure 1. Representative ethidium bromide stained gel of polymerase chain reaction (PCR) amplification products of bcl-2 from normal lymphocytes. Lane 1 is a control without bcl-2 primers, lanes 2–7 show the use of increasing amounts of cDNA. (b) Plot of areas under the curve as measured by high performance liquid chromatography (HPLC) analysis summarising the same information as shown in (a). An almost perfect correlation between the expected and the measured amount of PCR product is demonstrated, indicating linearity of amplification in the used range of cDNA amounts (*x* axis = volume of cDNA; *y* axis = area under the curve of HPLC-measured amplified fragment).

for 1 min and 72°C extension for 1 min and then by 28 cycles of 94°C for 50 sec, 55°C for 40 sec and 72°C for 40 sec, followed by 94°C denaturation for 1 min and 10 min at 50°C.

High performance liquid chromatography (HPLC) quantification

HPLC quantification of PCR products was performed as described previously [14, 17, 20], using the TSK DEAE-NPR column (Perkin Elmer Europe, Rotlereus, Switzerland) and determining the optical density at 260 nm. The ratio of the obtained areas under the curve (AUCs) *p53*/*β2-M*, *c-myc*/*β2-M* and *bcl-2*/*β-actin* indicated the relative expression of *p53*, *c-myc* and *bcl-2* genes in the colorectal tumour metastases, which was then compared with *p53*, *c-myc* and *bcl-2* relative expression in samples of normal colon mucosa. The linearity of amplification was confirmed by amplifying cDNA for every sample in each reaction at two different concentrations and by checking the linear range of PCR amplification for each gene in each PCR reaction using a standard control. At least two separate amplifications were performed for each sample. Ethidium bromide stained gels of PCR amplification products were run for *bcl-2* and *β-actin* confirming the linearity and accuracy of HPLC quantification. Figure 1(a) shows a representative ethidium bromide stained gel and Figure 1(b) a plot of the AUCs following HPLC quantification of PCR products obtained by amplification of various amounts of control normal lymphocyte mRNA using the *bcl-2* primers. The almost perfect correlation between the expected and the measured amount of amplified *bcl-2* fragment in the linear range of the PCR procedure method ($R^2 = 0.9957$; Figure 1b) emphasises the reliability of our semiquantitative PCR. In a previous publication, we confirmed that the ratio of the *β actin* and *β2-M* housekeeping genes was constant in the assayed tumour samples and controls and that *p53* and *c-myc* relative ratios were not significantly affected by the use of either *β actin* or *β2-M* as reference genes [14]. The comparison of *bcl-2* and *p53* or *c-myc* relative ratios of expression was thus reliable using either *β-actin* or *β2-M* as a reference gene.

Statistical analysis

For statistical analyses of independent samples we used the Mann–Whitney rank-sum test. To identify associations between two variables within a single group of samples we performed the Spearman rank correlation test. P values < 0.05 were considered significant.

RESULTS

bcl-2 *expression*

To determine *bcl-2* mRNA expression, the ratio of *bcl-2* and *β-actin* gene cDNA amplification product was calculated for each sample. Five specimens of normal colonic mucosa were analysed and the mean of the ratios used as the standard (1.0 U) for all PCR experiments measuring the expression of *bcl-2* mRNA. The relative *bcl-2* mRNA expression of the 21 colorectal tumour metastases analysed is shown in Table 1. On average, tumour tissue expressed less *bcl-2* mRNA than normal mucosa (mean 0.45 U, median 0.36 U, range 0.09–1.34 U, $P < 0.0001$), Decreased expression was defined as *bcl-2* mRNA expression less than half of normal mucosa (= 1 U). Of 21 tumours, 14 (67%) had decreased *bcl-2* mRNA expression. For 18 of 21 tumour samples, histopathological grading was available. No correlation existed between the level of mRNA expression and the tumour grade (Spearman rank test, $r = 0.017$, confidence interval -0.47–0.49, $P = 0.95$).

Correlation of bcl-2 *mRNA expression to* p53 *and* c-myc *expression*

For 19 of the 21 colorectal tumour metastases included in the study, *p53* and *c-myc* expression had previously been determined [15] and the relative values to normal colon mucosa are listed in Table 1. Overexpression was defined as a

Table 1. *Expression of the* bcl-2, p53 [14] *and* c-myc [15] *genes in metastases of colorectal cancer relative to normal colonic mucosa* (= 1 U)

UPN	*bcl-2* expression	*c-myc* expression	*p53* expression	PAb1801 staining	*p53* mutation*	Codon	Exon	Mutation	
1	0.29	8.80	5.20	++	+	266	8	G→A	Gly→Arg
2	0.29	9.10	5.00	+++	+	266	8	G→A	Gly→Arg
4	0.40	8.9	3.10	++	+	248	7	G→A	Arg→Gln
8	0.68	2.00	0.90	+	+				
19	0.40	4.10	3.00		+	257	7	T→C	Leu→Pro
21	0.11	1.80	3.70		–				
24a	0.97	1.50	2.50		–				
25	0.66								
26	0.24								
27	0.30	8.00	0.60		–				
28	0.76	1.60	0.60	++	–				
32	0.40	1.40	0.80	+	–				
34	0.15	1.80	2.10		+	245	7	G→A	Gly→Ser
35	0.10	4.10	3.10	+	+	237	7	Del	Met
43	0.09	6.50	3.00	+	+	248	7	G→A	Arg→Gln
45	0.36	1.50	2.40	–	+	26–32	3	Del	STOP
50	0.22	8.60	4.20	++	+	273	8	G→A	Arg→His
51a	1.34	1.50	1.70	+++	+	175	5	G→A	Arg→His
65	0.65	1.40	1.00	–	–				
66	0.22	2.20	2.30	+++	+	245	7	G→A	Gly→Ser
72	0.78	1.50	1.10	–	+	158	5	C→T	Arg→Cys

*, *p53* mutation; +, mutated; –, wild-type [14]. Immunohistochemical staining with the anti-p53 monoclonal antibody PAb1801: +++, intense staining in most cells; ++, intermediate staining or strong staining of 50–70% of cells; +, weak; –, absent staining [14]. UPN, universal patient number.

more than 2-fold elevation of *p53* mRNA or *c-myc* expression over that of normal colonic mucosa. The *p53* mutational status determined by direct sequencing in 19 of the 21 samples and the immunohistochemical quantification of p53 protein levels with the monoclonal antibody PAb1801 in 14 of the 21 samples are also summarised in Table 1. *p53* mRNA was overexpressed in 12 of 19 tumours (63%, mean 3.3 U, median 3.05 U, range 2.1–5.2 U). Tumours harbouring *p53* gene mutations were significantly ($P = 0.02$) more likely to overexpress p53 mRNA in relation to normal mucosa (relative expression levels > 1) and to display immunohistochemical evidence of p53 protein accumulation ([14], Table 1).

A significant correlation existed between *p53* overexpression and reduced *bcl-2* expression (Mann–Whitney $P = 0.0052$, Spearman rank $P = 0.021$; Table 1 and Figures 2a, 3a). We found the same reciprocity of *bcl-2* and *p53* expression in a separate analysis of the 13 tumour samples known to harbour *p53* mutations (Mann–Whitney $P = 0.007$, Spearman rank $P = 0.02$; Table 1 and Figures 2b, 3b).

In 9 of 19 samples, *c-myc* was overexpressed (47%, mean 6.7 U, median 8 U, range 2.2–9.1 U). *bcl-2* and *c-myc* were again inversely correlated (Mann–Whitney $P = 0.022$, Spearman rank $P = 0.025$; Table 1 and Figures 4, 5).

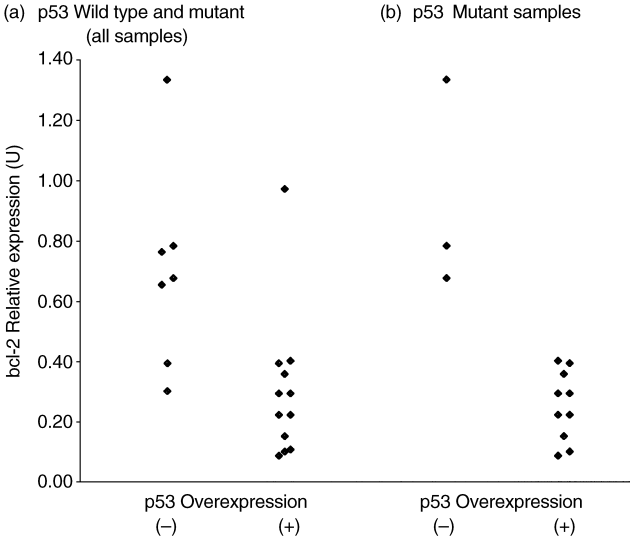

Figure 2. Expression of the *bcl-2* gene as a function of expression of *p53* for all samples ($n = 19$) and for *p53* mutant samples ($n = 13$). +, overexpression of *p53*; –, no overexpression. (a) Means of *bcl-2* expression for all tumours: 0.70 U (–) and 0.30 U (+), $P = 0.0052$. (b) Means of *bcl-2* expression for tumours carrying *p53* mutations: 0.93 U (–) and 0.25 U (+), respectively, $P = 0.007$.

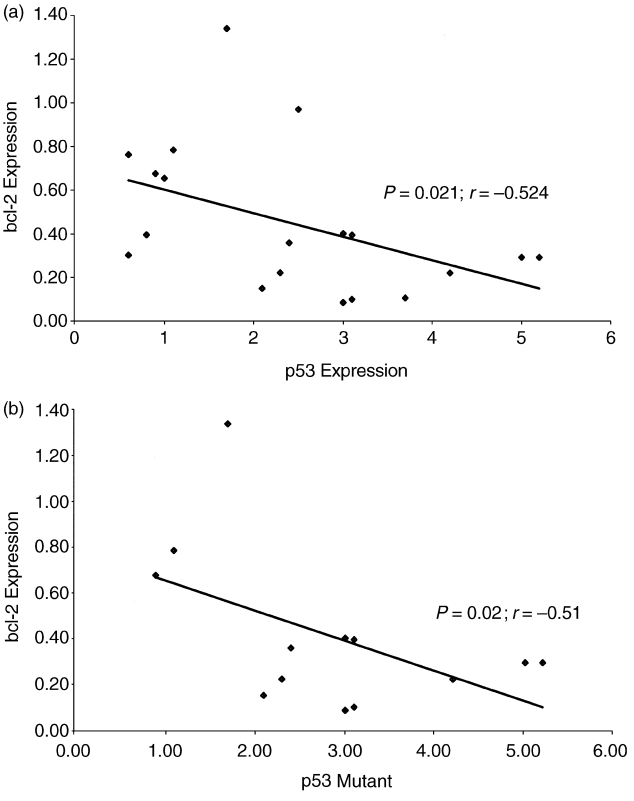

Figure 3. Correlation between the expression of *bcl-2* and *p53* (1 U = normal colonic mucosa) (a) for all tumour samples ($n = 19$); (b) for tumour samples carrying p53 mutations ($n = 13$).

Ki-ras *oncogene mutations and* bcl-2 *expression*

16 of 21 samples had previously been analysed for *ki-ras* oncogene mutations; 10 samples contained wild-type *ki-ras*, while six carried mutations localised to the second position of either codon 12 or 13 [16]. There was no correlation between

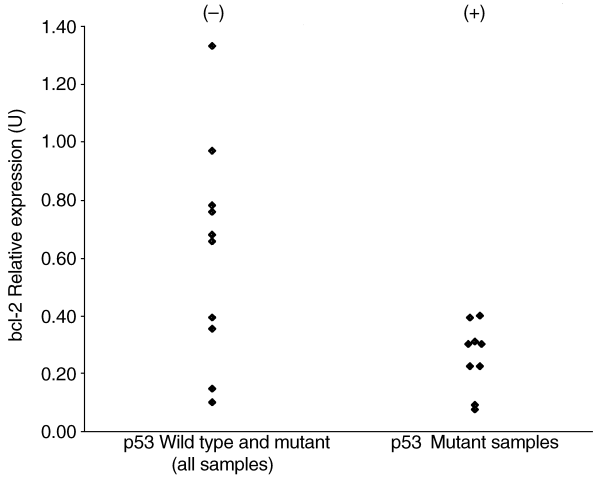

Figure 4. Expression of *bcl-2* as a function of expression of *c-myc*. +, overexpression of *c-myc*; –, no overexpression (means 0.62 U and 0.26 U, respectively, $P = 0.022$).

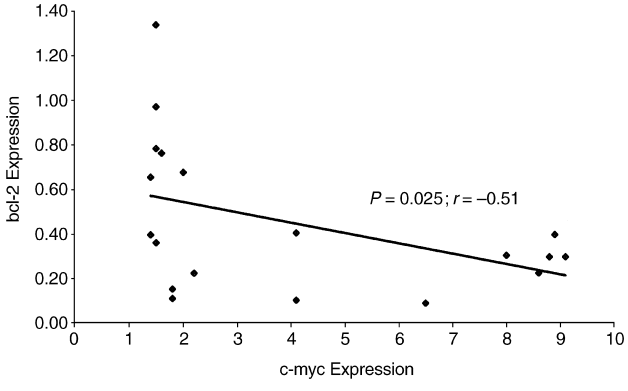

Figure 5. Correlation between the expression of *bcl-2* and *c-myc* (1 U = normal colonic mucosa).

ki-ras mutation and *bcl-2* mRNA expression ($P = 0.98$, data not shown).

DISCUSSION

Compared with colonic mucosa, 20 of 21 specimens of colorectal cancer metastases showed reduced levels of *bcl-2* mRNA (mean 0.45 U, $P < 0.0001$) and in 14 of 21 tumour specimens, mRNA expression was reduced to less than 50% of normal mucosal controls. Only one of the 21 tumours had *bcl-2* mRNA levels higher than normal mucosa. There was no correlation between tumour grade and *bcl-2* mRNA expression. Our findings of reduced *bcl-2* mRNA levels in colorectal cancer metastases are in agreement with immunohistochemistry studies [7–9] reporting fewer bcl-2 positive cells and weaker staining in adenocarcinomas than normal mucosa. bcl-2 positive cells have been described as pathologically extending into parabasal and superficial mucosal layers in dysplastic colon mucosa and phenotypicaly normal mucosa immediately adjacent to adenocarcinomas [5, 8, 9], but not in colonic epithelium adjacent to colon cancer metastases [5]. Similarly, bcl-2 immunostaining in non-neoplastic polyps does not differ from normal colon mucosa, but there is increased staining in premalignant adenomas [7, 8]. Sinicrope and colleagues [7] showed that apoptosis in colon epithelial cells (as measured by a histological apoptotic index) correlated inversely with the number of bcl-2 positive cells and the intensity of staining. bcl-2 accumulation in dysplastic colon mucosa cells is probably an early primary event in the development of colorectal cancer, conferring protection from apoptotic cell death and favouring the accumulation of genetic alterations required for colorectal tumorigenesis [1]. It would be intriguing if this mechanism were abandoned in advanced colon cancers, where *bcl-2* mRNA was shown by us and bcl-2 protein by others, to be strongly reduced. In this setting, our finding of reciprocal expression of *bcl-2* and *p53* or *c-myc* is particularly interesting, since both the latter genes are also regulators of apoptosis [21]. Overexpression of *p53* or *c-myc* (defined as over a 2-fold increase compared with normal mucosa) was consistently associated with very low *bcl-2* mRNA levels (Figures 2–5). *p53* mRNA overexpression correlated with the presence of *p53* mutations and p53 protein accumulation as determined by immunohistochemistry. When the 13 samples which harboured *p53* mutations were analysed separately regarding *bcl-2* mRNA expression, the same reciprocity was observed. High levels of mRNA for *bcl-2* and *p53* or *c-myc* seemed mutually exclusive. *p53* may modulate apoptosis via multiple pathways. Wild-type p53 protein and some mutated forms have been reported to transcriptionally downregulate *bcl-2* expression through a negative response element in the *bcl-2* 5′ flanking region [22]. *p53* overexpression has also been shown to upregulate Bax, a 21 kDa protein with *bcl-2* sequence homology that is in turn a dominant inhibitor of *bcl-2* [23]. Both mechanisms may account for the significant inverse relationship between *p53* and *bcl-2* reported at the mRNA level in 19 metastases ($P = 0.02$), which also holds true for the 13 tumours which carried a mutated *p53* ($P < 0.05$). Others [6, 9] have confirmed the same reciprocity through immunohistochemical methods. Watson and colleagues found the expression of both *bcl-2* and *p53* a rare occurrence and in three double stained neoplasms, *p53* and *bcl-2* were expressed reciprocally, although the *p53* mutational status was unknown [9]. An inverse correlation between *p53* and *bcl-2* was also recently reported for non-small cell lung cancer [24]. An alternative explanation for low *bcl-2* levels in advanced colorectal cancers may reside in allelic loss, given the *bcl-2* gene position close to the *DCC* gene on chromosome 18q, a region lost in up to 70% of colorectal carcinomas [1]. The significant reciprocity of *bcl-2* and *c-myc* expression described in 19 advanced colon cancers has not been observed previously. Evidence exists to suggest that *bcl-2* may co-operate with *c-myc* to achieve a malignant state in lymphoid tissues [25] and the interaction of *bcl-2* and *c-myc* has been shown to inhibit *p53*-mediated cell cycle arrest and apoptosis [26]. We recently showed that *c-myc* expression and mutated, but not wild-type, *p53* expression are strongly correlated in colorectal cancer [15]. Wild-type *p53* represses *c-myc* transcription [27], thus inhibiting *c-myc*-induced cellular proliferation; in cells where *c-myc* overrides *p53*-mediated G1 arrest and drives cells into the S phase, wild-type *p53* then triggers apoptosis [28]. Mutated p53 protein, however, loses the ability to inhibit c-myc accumulation or to induce apoptosis. A *bcl-2* independent pathway of evading apoptosis is thus established, obliterating the need for *bcl-2* expression as a safeguard against programmed cell death. Because of the small number of samples, we could not establish whether the inverse correlation between *bcl-2* and *c-myc* expression is likely to be due to the reported interdependence of *c-myc* with mutated *p53*, or whether it is independently mediated and this question needs further investigation.

A number of publications have investigated the prognostic significance of *bcl-2* oncoprotein expression in colorectal cancer. Barreton and colleagues correlated bcl-2 immunohistological staining with programmed cell death index, p53 accumulation and the proliferation index Ki-67 in 44 adenomas and 95 carcinomas [29]. bcl-2 was detected in 86% of adenomas and 67% of carcinomas. When present, *bcl-2* expression was very significantly correlated with a low programmed cell death index. Furthermore, in the univariate, but not multivariate, analysis, patients with tumours displaying bcl-2 positivity or bcl-2 positive/p53 negative had longer disease-free survival. In contrast, another recent immunohistochemical study [30] of 48 archival colorectal cancer samples found that by univariate analysis bcl-2 overexpression was associated with histologically less differentiated tumours and poorer overall survival. Oefner and colleagues [31] examined the prognostic value of bcl-2 oncoprotein expression in a series of 104 colorectal carcinomas. In 55 tumours no bcl-2 was detectable immunohistochemically, and 22 of 104 tumours had 5% or more cells staining for bcl-2. Low levels of bcl-2 staining correlated significantly with larger tumours and poorer survival and in multivariate analysis bcl-2 expression remained an independent prognostic parameter. Manne and colleagues [32] recently immunohistochemically determined bcl-2 and p53 expression in 134 colorectal adenocarcinomas and confirmed that bcl-2 expression, present in 50% of cases, was significantly associated with better survival. Nuclear p53 accumulation correlated with poorer outcome, and patients whose tumours expressed p53, but not bcl-2, did worst. An inverse correlation between bcl-2 and p53 was noted, but multivariate analysis showed that the two parameters remained independent prognostic factors in this series. An inverse correlation for these two oncoproteins and a survival advantage in cancers expressing higher levels of bcl-2 was shown for other primary sites, including the lung [24]. Some of the discrepancies in the studies cited above may be

due to the use of different immunohistochemical techniques and detection thresholds for bcl-2 and p53, and sample size. Furthermore, p53 overexpression determined by immunohistochemistry has been shown to correlate only moderately with allelic loss or *p53* gene mutation and while the latter two have been shown to be useful markers of prognosis in colorectal cancer, results of studies using p53 overexpression have been more ambiguous [10, 11, 33]. Data on *bcl-2* mRNA expression in colorectal cancer do not yet exist to our knowledge.

The results of our study suggest that *bcl-2*-mediated apoptosis inhibition is not a relevant pathway by which advanced colorectal carcinoma cells avoid programmed cell death, although available immunohistochemical evidence indicates that it may be an important early mechanism by which dysplastic colonic mucosal cells can accumulate genetic alterations and escape apoptosis, allowing colorectal cancers to become established. The low levels of *bcl-2* in metastatic colon cancer may represent allelic loss of the 18q chromosome, the reciprocity in *bcl-2* and *p53*/*c-myc*, however, seems to indicate downregulation of *bcl-2* by factors that come into play later in the molecular progression to advanced cancer and to which *bcl-2* may delegate its apoptosis inhibiting function. This may explain why low levels of bcl-2 protein may be associated with poorer prognosis and more aggressive colorectal tumours.

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