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Bcl-2 Expression in Colorectal Tumors

Evidence of Different Pathways in Sporadic and Ulcerative-Colitis-Associated Carcinomas

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Colorectal cancers (CRCs) differ in their age at presentation, distribution, histological features, and prognosis. If tumor biology reflects genetic events, these tumors might be expected to show differences in their genetic pathways. In this study, we investigated the role of Bcl-2 in the development of three different tumor groups. Using markers at eight different microsatellite loci, we characterized one group of 34 left-sided sporadic CRCs as replication error negative (RER$^-$) and another group of 18 left-sided sporadic CRCs as replication error positive (RER$^+$). These tumors, together with a third group of 22 left-sided ulcerative-colitis-associated CRCs (UCACRCs), were then examined by immunohistochemistry for Bcl-2 overexpression. Of 34 of the RER$^-$ tumors, 21 (62%) and 10 of 18 (56%) of the RER$^+$ tumors were positive for Bcl-2 overexpression. In contrast, only 5 of 22 (23%) of the UCACRCs showed similar overexpression. Our results show a significantly lower frequency of Bcl-2 overexpression in UCACRCs as compared with sporadic CRCs ($P < 0.005$) but no difference between sporadic left-sided RER$^+$ and RER$^-$ CRCs. These data provide additional evidence that UCACRCs may develop along a pathway that is different from that of sporadic CRCs. (Am J Pathol 1996, 149:1719–1726)

It is almost universally accepted that carcinogenesis is a multistep process.[1] At each step, a mutation occurs that results in either activation of an oncogene or inactivation of an oncosuppressor gene.[2] The net result of these events is escape from growth control with the acquisition of an invasive and ultimately metastatic phenotype. The necessary mutations vary among different tissue types, depending on the controls to which the cells within a particular tissue are subject.[3] However, even within a single tissue type, there may be a variety of alternative genetic pathways leading to tumor formation. If cells can use different groups of mutations to overcome the same growth controls, then some differences in tumor characteristics might be expected as a reflection of the effect of these mutations. Conversely, morphologically and biologically distinct tumors arising in any single tissue may be predicted to show differing patterns of mutations. In this study, we examined three groups of colorectal cancers (CRCs): left-sided sporadic CRCs without replication errors (RER$^-$ CRCs), left-sided sporadic CRCs with replication errors (RER$^+$ CRCs), and left-sided ulcerative-colitis-associated CRCs (UCACRCs).

Sporadic CRCs mostly arise distal to the splenic flexure and are mainly RER$^-$ (see below). Usually they pass through phases of progressively more severe adenomatous dysplasia before acquiring invasive properties. Some of the mutations leading to the development of sporadic CRCs are described in the model proposed by Fearon and Vogelstein and can be correlated to some degree with histological appearances.[4]

RERs can occur as a result of defects in DNA mismatch repair (MMR).[5] RERs are most easily ob-

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served at nucleotide repeat sequences termed microsatellites and can result in nucleotide insertions or deletions at these sites.[6] The consequent cell-to-cell variation in the size of the microsatellites is termed microsatellite instability. RERs are found in the cancers of patients with the hereditary non-polyposis colorectal cancer syndrome. In these cases, germline mutations in one of four MMR genes result in early development of RER^+ CRCs.[7,8] Ten to twenty percent of sporadic CRCs are RER^+[7–9] and therefore presumably have somatic loss of MMR. In comparison with sporadic RER^- CRCs, sporadic RER^+ CRCs present at an earlier age,[10] are more usually right-sided (ie, proximal to the splenic flexure), and more often show mucinous differentiation.[9]

The development of UCACRCs, the third type of tumor, is related to the age of onset and to the extent and duration of ulcerative colitis.[11] UCACRCs usually present at an earlier age than sporadic CRCs[12] and are more frequently multiple with diffuse macroscopic appearances.[13] In contrast to the polypoid, well circumscribed adenomas that usually form the precursors of sporadic CRCs, the dysplastic precursor lesion of UCACRCs is flat and diffuse, often involving large areas of the large bowel.[14] The areas of dysplasia are distributed throughout the large bowel although UCACRCs are predominantly left-sided.[15] There is some evidence that *APC* and K-*ras* mutations occur less frequently and that aneuploidy and p53 mutations occur earlier in UCACRCs than in sporadic CRCs.[16–19]

The *bcl-2* gene product is a 26-kd cytoplasmic protein that is thought to prevent apoptosis.[20,21] The role of Bcl-2 in malignancy is best described in follicular lymphomas in which the chromosomal translocation t(14; 18) (q32;q21) results in the juxtaposition of the *bcl-2* proto-oncogene and the immunoglobulin heavy chain gene.[22,23] The resulting constitutive overexpression of Bcl-2 protein immortalizes lymphocytes to allow accumulation of other mutations and eventual malignant transformation. Bcl-2 has also been shown to have a role in the biology of several normal epithelial tissues, and protein overexpression has been reported in tumors arising from these epithelia.[24–27] In the normal large bowel, Bcl-2 expression is seen in the proliferative compartments at the base of the crypts.[28] Overexpression of this protein has been reported in a high proportion of both colorectal adenomas and CRCs, and as such, Bcl-2 overexpression has been deemed an early event in the development of these tumors.[29,30]

The aim of this study was to examine Bcl-2 expression in three different types of colorectal cancer to ascertain whether there were differences in expression among these tumor groups and thus add weight to the view that they each may have a disparate oncogenic basis.

Materials and Methods

Frozen tissue from 98 cases of sporadic left-sided CRC was retrieved from the tissue bank at the Colorectal Unit of the Imperial Cancer Research Fund and examined for microsatellite instability. Cases with a family history of cancer, familial adenomatous polyposis, or inflammatory bowel disease were specifically excluded. There were 18 cases that were RER^+, and all of these cases, together with 34 RER^- cases, were selected for immunohistochemical analysis. In addition, 22 left-sided UCACRCs were selected from the archives of the Pathology Department of St. Mark's Hospital. These also underwent polymerase chain reaction (PCR) analysis for microsatellite instability and, together with the 52 cases of sporadic CRC, were subjected to immunohistochemical analysis. In total, 74 cases were studied for microsatellite instability and Bcl-2 expression.

PCR and Microsatellite Instability

DNA from the sporadic tumors was extracted from paired samples of frozen tumor and normal tissue using standard methods. Frozen tissue was not available for the UCACRCs, and PCR analysis was performed on formalin-fixed, paraffin-embedded tissue microdissected from freshly cut sections. Two 4-μm sections from each case were de-waxed and stained lightly with hematoxylin. Areas of tumor and normal tissue were easily identified in the dry sections, and one section each was used to provide normal and tumor tissue. A 10-μl volume of distilled water was placed on the area of the slide to be microdissected, and tissue was scraped into the water using a sterile 25-gauge needle. The water containing the tissue fragments was pipetted off the slide and added to an equal volume of digestion mix that, after addition of the tissue, consisted of proteinase K (500 μg/ml) and Tween 20 (0.5%) in 20 mmol/L Tris/HCl (pH 8.3).[31] After overnight digestion, samples were boiled for 10 minutes to inactivate the proteinase K, and 1 μl of the digest was used in the PCR reaction.

Microsatellite instability was assessed at eight dinucleotide repeat markers. PCR reactions were performed on each tumor/normal pair using 50 to 250 ng of DNA template in a final volume of 50 μl. The reaction mixture contained 1X standard PCR buffer (Promega, Southampton, UK), 1.5 mmol/L

Mg^{2+}, 0.5 μmol/L dNTPs, 0.4 μmol/L of each specific oligonucleotide primer and 1 U of *Taq* polymerase. The thermal cycling protocols were as follows: for D11S968, D11S901, D11S1313, and D6S434, 94°C for 1 minute, 94°C for 1 minute/55°C for 1 minute/72°C for 1 minute 35 times, and 72°C for 5 minutes[32]; for D11S29, 94°C for 1 minute, 94°C for 1 minute/50°C for 1 minute/72°C for 1 minute 30 times, and 72°C for 10 minutes[33]; for NCAM and D16S520, 94°C for 1 minute, 94°C for 1 minute/50°C for 1 minute/72°C for 1 minute 30 times, and 72°C for 5 minutes (modified from Telatar et al[34]); for DRD2, 94°C for 1 minute, 94°C for 30 seconds/58°C for 30 seconds/74°C for 30 seconds 30 times, and 74°C for 5 minutes.[35] After heating to 90°C for 5 minutes, the PCR products underwent electrophoresis under denaturing conditions on a 6% acrylamide sequencing gel (Sequagel) for 2 to 4 hours. DNA was blotted onto Hybond N+ membranes (Amersham, Little Chalfont, UK), and PCR products were detected by the enhanced chemiluminescence technique (Amersham), using a randomly elongated oligonucleotide primer as a specific probe for each locus. Membranes were exposed to Hyperfilm (Amersham) for up to 60 minutes to allow visualization of PCR products. Extra bands differing by multiples of 2 bp from their normal counterparts at each locus were noted and used to classify tumors as RER^+ (see below for definition).

Tumor Pathology

The histology of all tumors was reviewed by one pathologist (M. Ilyas), and the tumors were graded as well, moderately, or poorly differentiated CRCs according to degree of tubule formation and nuclear pleomorphism. Information about the stage at presentation of the tumors was obtained from the reports issued for the specimens.

Immunohistochemistry

All 74 tumors underwent immunohistochemical analysis for Bcl-2 overexpression. Fresh 4-μm-thick sections were cut from blocks of formalin-fixed, paraffin-embedded tumor tissue. Sections were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol and sections were then boiled for 30 minutes in an aluminum pressure cooker at 15 psi in sodium citrate buffer (0.01 mol/L, pH 6.0) to unmask antigen. Sections were incubated overnight at room temperature with the mouse monoclonal antibody Bcl-2 (Dako, High Wycombe, UK) at a dilution of 1:40. Bound primary antibody was detected using the labeled streptavidin system, with a second and third layer (each applied for 1 hour in a dilution of 1:200) of biotinylated polyclonal rabbit anti-mouse antibody (Dako) and horseradish-peroxidase-labeled streptavidin (Dako), respectively, and diaminobenzidine as the chromogen. Negative controls (performed by using phosphate-buffered saline instead of the primary antibody) were included in every experiment. Normal epithelium, myenteric nerve plexi, and lymphocytes in the sections served as internal positive controls.

The immunohistochemistry was reviewed independently by two pathologists (M. Ilyas and A. M. Hanby) blinded to the categorization of the tumors. Given the heterogeneity of the Bcl-2 staining, a dual scoring system was used, giving equal weight to both the intensity of staining and the proportion of positively stained tumor cells. Intensity of staining was scored on a scale of 0 to 3, with 3 being equivalent to the intensity seen in the proliferating cells in the base of crypts. The extent of staining of each tumor was scored semiquantitatively from 0 to 3 using the following scale: 0, no staining at all in the tumor; 1, <25%; 2, 25 to 50%; 3, >50% of cells stained. The scores of both reviewers were then added, and scores ranged between 0 and 12. Those cases with a score of 8 or more were called positive for Bcl-2 overexpression. A score of 8 was selected as the cut-off point because it was equivalent to moderate staining of over one-quarter of the tumor cells.

Results

PCR

We classified the sporadic tumors as RER^+ on the basis of extra bands at one or more loci (Figure 1). We subdivided these tumors into RER+1 (indicating instability at one microsatellite marker only) or RER+2 (indicating instability at two or more microsatellite markers). In the RER+1 group, microsatellite instability was accepted only when more than one new band was seen. Eighteen sporadic tumors were deemed RER^+. Nine of these were RER+1 and nine were RER+2. Thirty-four of the sporadic tumors were deemed RER^-. Of the UCACRCs, three of twenty-two (14%) were RER^+, all three cases showing instability at more than one locus (RER+2). Given the fact that we regard UCACRCs as a separate group of tumors, together with the small numbers of UCACRCs with microsatellite instability, all of the

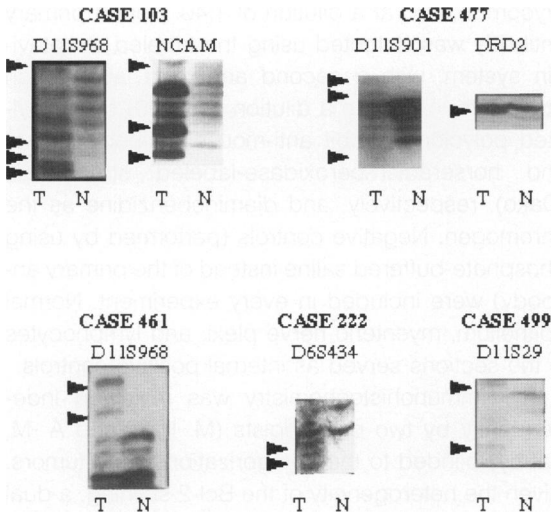


Figure 1. *Examples of microsatellite instability.* Arrows *show the positions of extra alleles in tumor (T) samples in comparison with normal (N) tissue. Other extra alleles can be seen as fainter bands but are not arrowed. Two tumors are unstable at two of the seven loci (cases 103 and 477), and three are unstable at one locus (cases 4461, 222, and 499).*

UCACRCs were analyzed together as a single group.

Tumor Pathology and Immunohistochemistry

Table 1 shows the histological grade, Dukes' stage, and proportion of mucinous tumors in each of the three tumor groups. There was no significant difference among the RER^+ CRCs, RER^- CRCs, or UCACRCs regarding these features.

The results of the immunohistochemistry are given in Figure 2, showing the distribution of the results in each group of tumors. In all of the cases, the normal epithelium showed strong staining for Bcl-2 at the crypt bases with a gradient of diminishing intensity along the crypt axis (Figure 3a). Faint staining was occasionally still visible at the top of the normal crypts. No evidence of a field change alteration in Bcl-2 expression was observed in any of the groups, with the overall intensity and localization remaining constant in the ostensibly normal bowel both near to and remote from to the tumors. Lymphocytes in the lamina propria and the Peyer's patches stained with equal intensity to that of the epithelial cells in the bases of the crypts. Staining of the tumors was generally heterogeneous, ranging from completely negative staining (intensity 0, extent 0; Figure 3b) through all degrees of intermediate staining (eg, focal intense staining (intensity 3, extent 1; Figure 3c) and extensive pale staining (intensity 1, extent 3) to extensive intense staining (intensity 3, extent 3; Figure 3d). Using our criterion that a total score of 8 represents positive staining, 21 of the 34 (62%) RER^- sporadic CRCs and 10 of 18 (56%) of the sporadic RER^+ tumors were positive for Bcl-2 overexpression. However, only 5 of the 22 (23%) UCACRCs were positive for overexpression. Statistical analysis showed that there was no difference in Bcl-2 expression between the sporadic RER^- and sporadic RER^+ tumors. This remained true when the RER+2 group alone was compared with the others (ie, RER^- plus RER+1). There was, however, a significantly lower frequency of positive Bcl-2 staining in the UCACRCs compared with the sporadic CRCs ($\chi^2 = 8.4$, $P < 0.005$). Each of the individual features of the immunohistochemistry (ie, intensity alone, extent alone, and total score) showed a significant difference between the sporadic tumors and the UCACRCs (Figure 2). Although the number of RER^+ UCACRCs was small, Bcl-2 expression in these tumors was unremarkable when compared with the RER^- UCACRCs.

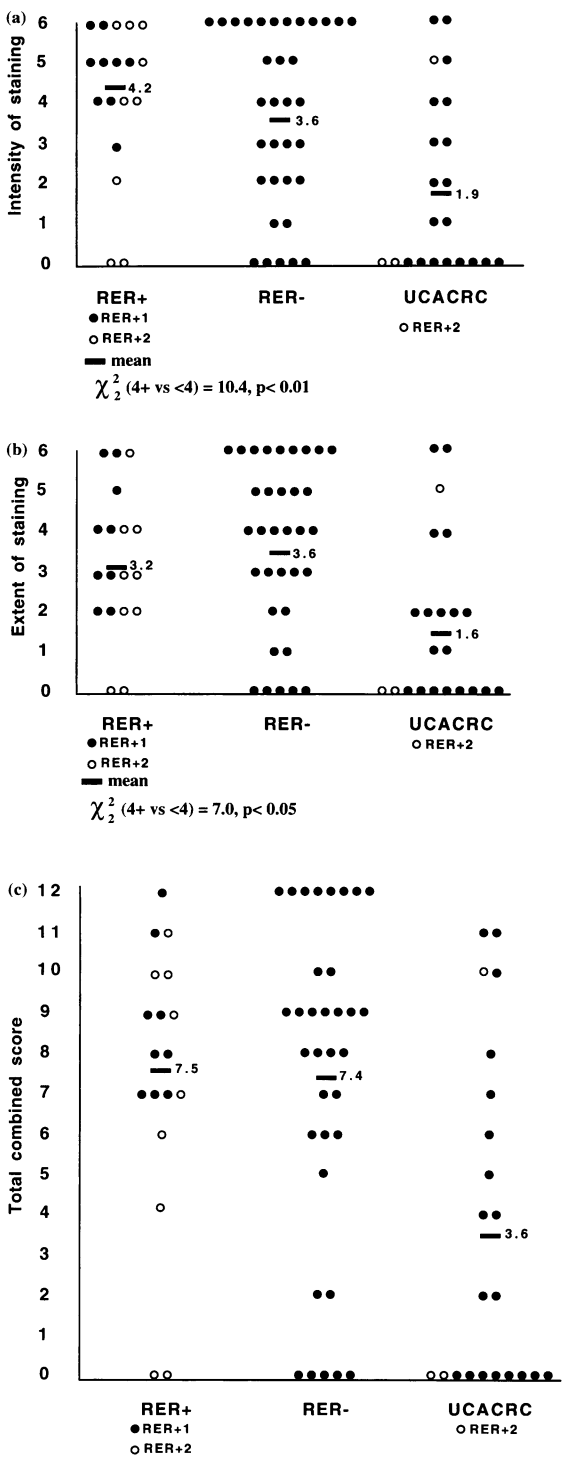
Discussion

The Fearon and Vogelstein model is a paradigm for carcinogenesis.[4] Encompassed within this model is the stepwise acquisition of mutations, which, to some degree, is reflected in histological appearances. The

Table 1. *Pathological Characteristics of Three Different Types of Colorectal Cancers*

	RER^+ (n = 18)	RER^- (n = 34)	UCACRC (n = 22)
Tumor differentiation			
Well differentiated	0	5 (14%)	2 (9%)
Moderately differentiated	16 (88%)	20 (60%)	15 (68%)
Poorly differentiated	2 (12%)	9 (26%)	5 (22%)
Dukes' stage			
A	2 (12%)	4 (11%)	5 (23%)
B	8 (41%)	21 (63%)	11 (50%)
C	8 (47%)	9 (26%)	6 (27%)
Mucinous tumors	1 (6%)	4 (11%)	5 (23%)

All tumors were left-sided (ie, distal to the splenic flexure).



model stresses that the total accumulation of mutations is the most important factor in tumor development although mutation selection appears to be nonrandom at each step. If the selected mutations control the clinicopathological features of any individual tumor, it is feasible that different sets of mutations (between tumors) may lead to differences in tumor characteristics. Thus, tumors with different characteristics may be supposed to have different mutations.

The role of Bcl-2 in the development of CRCs is uncertain. It is normally expressed in the bases of the crypts where it is thought to prevent apoptosis in the stem cell compartment.[28] In two studies, there has been demonstration of high Bcl-2 overexpression in sporadic adenomas and carcinomas.[29,30] In another study, performed on frozen tissue, correlating Bcl-2 expression with detection of apoptosis by *in situ* end-labeling techniques, abnormal patterns of Bcl-2 expression and a lower rate of apoptosis were seen more often in villous adenomas than tubular adenomas.[36] These studies suggest that Bcl-2 is indeed involved in the prevention of apoptosis in large bowel epithelium and that, if overexpression is important in the carcinogenetic process, it probably occurs as an early event.

In this study, we investigated the importance of Bcl-2 overexpression in the development of three distinct types of colorectal tumor. The tumor groups had a similar pathological profile in terms of differentiation and Dukes' stage at presentation. We found immunohistochemical overexpression of Bcl-2 in 60% of left-sided sporadic RER^- CRCs and 58% of left-sided sporadic RER^+ CRCs. That there was no difference in Bcl-2 expression between sporadic RER^- and RER^+ tumors is not an unexpected finding. Even in hereditary nonpolyposis colorectal cancer cases, where there is a germline mutation in one of the MMR genes, loss of MMR function may not occur until after adenomas have developed.[37,38] As apparent Bcl-2 overexpression is observed in the early phases of tumor development, then both types of tumor should show similar patterns of expression. Alternatively, if overexpression of Bcl-2 protein actually has a role in the later evolution of tumors, our

Figure 2. *Graphical representation of the results of the immunohistochemistry showing distribution of each of the features assessed in the different tumor groups.* a: *Intensity of staining.* b: *Extent of staining.* c: *Summated score of* a *and* b. χ^2 *tests of association have been used to compare Bcl-2 expression in each of the three tumor groups, assuming that both RER+ 1 and RER+ 2 sporadic tumors are actually* RER^+. *Very similar results were obtained when only the* RER^{+2} *tumors were classed as true* RER^+ *tumors (data not shown). Wilcoxon rank sum tests for nonparametric data have been run along side the* χ^2 *tests for comparison. RER+ 1, replication errors at one locus; RER+ 2, replication errors at more than one locus.*

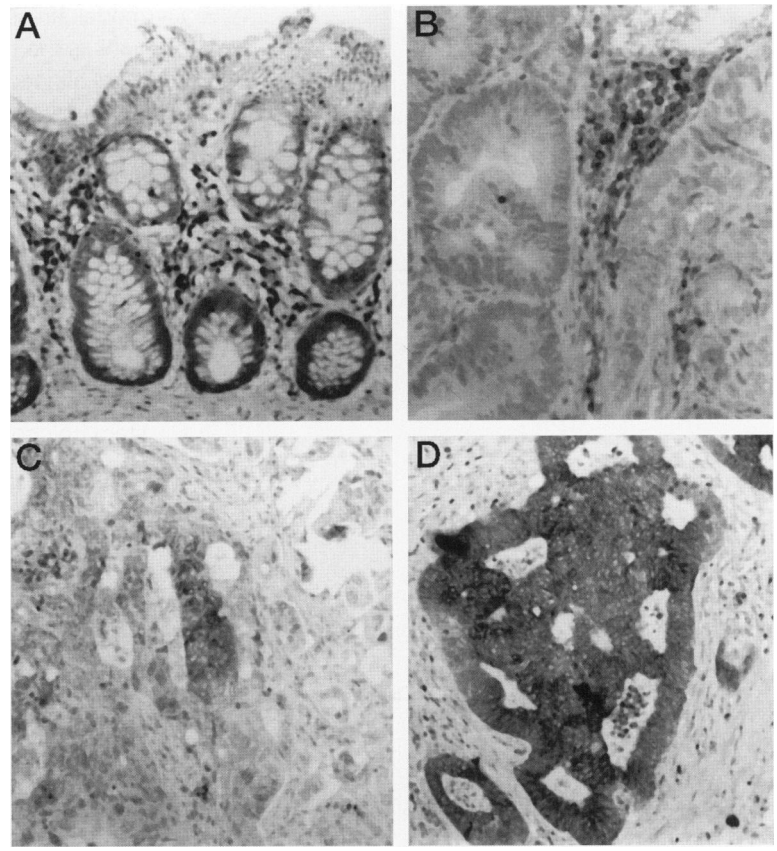


Figure 3. *Immunohistochemical staining for Bcl-2 in normal and neoplastic tissue.* A: *Normal epithelium shows strong expression in the base of the crypts with a gradient of decreasing expression toward the top of the crypts. The stromal lymphocytes are also strongly positive. Magnification, ×225.* B: *A tumor negative for Bcl-2 expression. The tumor cells are all negative whereas the infiltrating lymphocytes are positive. Magnification, ×288.* C: *A tumor showing heterogeneous expression of Bcl-2. There is a focus of strongly positive cells in the center and, apart from a small number of similar foci, there was no other staining. This tumor was classed as negative despite focal intense staining. Magnification, ×144.* D: *A tumor showing strong extensive expression of Bcl-2. Almost every cell was positive in this case. Magnification, ×115.*

findings support the possibility that, despite loss of MMR, RER^+ tumors progress along the same pathway as that of sporadic RER^- CRCs.

We found Bcl-2 overexpression in only five of twenty-two (23%) of our cases of UCACRCs. This is significantly lower than in sporadic CRCs ($P < 0.005$). This difference is not due to tumor differentiation as all three groups of tumors showed a similar spectrum of histological grading. Although there were more mucinous carcinomas in the UCACRC group, only one of these showed Bcl-2 overexpression. Exclusion of mucinous tumors did not affect the statistical significance of the results (data not shown). Three of twenty-two (14%) of the UCACRCs showed microsatellite instability, which is a lower frequency than that reported by one other group.[39] As the importance of defective MMR in the development of UCACRCs is not known and given the small number of RER^+ UCACRCs in this series, we decided to group all UCACRCs together. Additional studies are needed to ascertain whether RER^+ UCACRCs form a distinct biological subgroup.

Our data provide additional evidence that there are differences between UCACRCs and sporadic CRCs. Studies have shown, for example, that there is a lower incidence of mutations of the adenomatous polyposis coli (*APC*) and K-*ras* genes in these tumors.[16–19] In addition, p53 mutation has been shown by some studies to occur earlier in the development of UCACRCs.[40,41] This complements findings demonstrating aneuploidy in the early precursor lesions of UCACRC.[41–44] In cases where there is early gross change in nuclear content, loss of apoptotic pathways responsive to such changes in DNA (such as p53 mutation) would confer a survival advantage.[45] The mechanism of Bcl-2 inhibition of apoptosis is unknown, but it probably lies at the sites of localization in the cytoplasm.[46] It may therefore not provide a survival advantage in the early stages of the development of UCACRCs, in which the main apoptotic stresses probably arise from within the nucleus.

We found a lower frequency of Bcl-2 expression in sporadic carcinomas than reported in some other studies.[29,30] This may be a chance finding or may be due to differences in the criteria used for positive staining. Alternatively, the differences may be attributable to technical factors such as tissue fixation or sensitivity of the system employed to detect bound antibody. However, we feel that technical differences are unlikely to be an important factor, because there was strong staining in the normal epithelium and lymphocytes in all of our cases.

Our findings may result from mutations of the *bcl-2* gene itself. As with all studies of gene expression, however, the differences between sporadic CRCs and UCACRCs may actually reflect the effects of genes that modify *bcl-2* gene expression. Alternatively, rather than being an event of primary pathogenic importance, Bcl-2 overexpression may be an epiphenomenal event that changes in response to the features of the tumor cell population. For example, if Bcl-2 is expressed in proliferating colonic epithelial cells for reasons not actually associated with apoptosis, then a large proliferative compartment of a tumor will lead to extensive expression. Even if mutation of *bcl-2* itself does not cause the differences between UCACRCs and sporadic CRCs that we have observed, our results still suggest potentially important biological differences between these groups of CRC. Additional investigation is required to determine the cause of these differences.

In summary, we investigated Bcl-2 overexpression in three different groups of colorectal tumor. Our data show no difference between sporadic RER^+ and RER^- tumors and support the theory that these tumors develop along broadly similar carcinogenetic pathways. The results do show that UCACRCs have a significantly lower frequency of Bcl-2 expression than sporadic CRCs and thus provide additional evidence for divergence of the carcinogenetic pathways of this type of tumor.

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