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Immunohistochemical expression of bcl-2 in UICC stage I and III colorectal carcinoma patients: correlation with c-erbB-2, p53, ki-67, CD44, laminin and collagen IV in evaluating prognostic significance

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Background: The objective of this study was to evaluate the expression of bcl-2 in UICC stage I and stage III (Dukes's stage B and C) colorectal adenocarcinoma and to examine its association with clinicopathological features, c-erbB-2, p53, ki–67, CD44, laminin and collagen IV and long term outcome. Methods: Paraffin embedded specimens from 61 patients with UICC stage I (Dukes's stage B) and 39 patients with UICC stage III (Dukes's stage C) colorectal adenocarcinoma who were treated with surgery were assessed. We determined by immunohistochemistry the expression of bcl-2, c-erB-2, p53, ki–67, CD44, laminin and collagen IV with 5 year follow up. Results: Cytoplasmic staining of the bcl-2 gene product was seen in the tumour cells of 27 cases (27%). Expression of bcl-2 protein was unrelated to patient sex, age, tumour site or tumour grade, but was related to tumour stage (p=0.012). No significant association was demonstrated between bcl-2 and c-erbB-2, p53 or CD44. However, there was very strong evidence of correlation between bcl-2 staining and ki–67, laminin and collagen IV. There was a trend towards increased survival in patients whose tumours expressed bcl-2 protein. When a correlation between bcl-2 and the other markers had been made the positive expression of bcl-2 was beneficial. Conclusions: The results from this study would suggest that expression of bcl-2 appear to be useful in selecting a group of colorectal cancer patients with a better prognosis.

Introduction

Colorectal cancer results from a series of genetic events, which disorder the normal mechanisms controlling cell growth. The bcl-2 proto-oncogene is an inhibitor of apoptosis and may therefore permit the accumulation of genetic alterations propagating cell division and potentially contribute to tumour development [10]. The bcl-2 gene is located at chromosome 18q21 and its product is a 24kd protein localized to the nuclear envelope endoplasmic reticulum and mitochondrial membranes. The bcl-2 proto-oncogene was originally identified in the studies of t (14; 18) chromosomal translocation which occurs in most human follicular lymphomas [19] . This translocation juxtaposes the bcl-2 gene and the immunoglobulin heave-chain locus; consequently under the control of the immunoglobulin gene promoter, the bcl-2 protein is over expressed in these lymphomas [28]. bcl-2 has been proposed as the first example of a novel carcinogenesis pathway, acting through a block of cell death without affecting cell proliferation. Increased bcl-2 expression has also been reported in epithelial malignancies, e.g. carcinoma of the lung, thyroid, breast, stomach and ovaries. In the large bowel bcl-2 protein has been localized to the epithelial cells at the base of crypts, where stem cell proliferation takes place [30, 34].

The c-erbB-2 gene is located on human chromosome 17q21 and produces c-erbB-2 protein, which is an 185kDa-glycoprotein of the tyrosine kinase family. Over expression of c-erbB-2 is reported to be associated with malignant cell transformation and poor prognosis in several

carcinomas. The gene may promote metastasis in colorectal cancer by several mechanisms: a) over expression of its product, leading to abnormal proliferation potential via interaction with epidermal growth factor receptor, b) a higher extra-cellular matrix protease activity through c-erbB-2 up-regulated expression of urokinase – type plasminogen activator (uPA), c) an increased resistance to cytotoxic effects of tumor necrosis factor-alpha, released by activated macrophages [37].

p53 is a tumour suppressor gene that plays a key role in the control of the cell cycle. Cell proliferation is inhibited by p53 protein, which acts by arresting the cell cycle at the G1-s phase to allow DNA repair to take place. Loss of this activity may lead to neoplastic transformation. Alteration of this suppressor gene is a common event in colorectal carcinoma and has been associated with adverse postoperative outcome and poor survival [26].

ki–67 is expressed in cells actively engaged in the cell cycle and has also been used as a measure of proliferation in this patient population [1].

Recent studies have identified CD44 glycoproteins as potentially important components of tumour progression and the metastasis cascade [12, 14]. CD44 was originally described as a homing receptor on lymphocytes, mediating lymphocyte interactions with high endothelial venues [32]. Metastasizing tumor cells and recirculating (activated) lymphocytes save several properties including motility and invasive behavior involving reversible adhesive contacts, accumulation and expansion in draining lymph nodes and adhesion to vascular endothelium and extravasations. This analogy between circulating lymphocytes and tumour dissemination prompted the hypothesis that malignant cells might use molecules like CD44 for metastasis formation.

Laminins are a family of glycoproteins of the extra cellular matrix that function in the development and maintenance of cellular organization in the basement membrane. They regulate cell adhesion, migration, and differentiation [3]. This obviously has implications with regard to invasion and dissemination of malignant tumours of epithelial origin. Basement membranes play an important role in the development and maintenance of most tissues where they provide structural boundaries between specific cell types and connective tissues [15]. Depleted or discontinuous basement membrane structures have been reported for several types of gastrointestinal cancers, an observation often associated with poor prognosis.

Collagen type IV is the major structural protein of basement membranes, and several proteinases are known to attack and degrade this collagenous component.

Sinicrope et al [29] reported the first data concerning bcl-2 expression and apoptosis in colorectal tumorigenesis: in 17 of 24 (71%) colonic adenomas and 14 of 21 (67%) adenocarcinomas bcl-2 immunoreactivity could be detected. In most of the clinical studies, bcl-2 cytoplasmic expression correlated with a better prognosis [16]. Conversely, in some other studies bcl-2 correlation with poor survival has been reported [6]. The aim of this study was to investigate bcl-2 immunoreactivity in colorectal carcinoma and to determine its association with clinicopathological features, c-erbB-2, p53, ki–67, CD44, laminin and collagen IV and with long-term outcome.

Patients and Methods

Patients

A series of 100 patients underwent surgical resection for primary colorectal adenocarcinoma at the III Department of Surgery, Tzanio Hospital of Athens between 1995–1999. Cases of non-inherited polyposis colorectal cancer, familial adenomatous polyposis or ulcerative colitis and patients who died in the immediate postoperative period were excluded from the study. None of the patients had received pre- or postoperative radiotherapy or chemotherapy. Each patient was regularly followed up at 6 monthly intervals for a minimum of 5 years. Clinical staging was done on the basis of the UICC classification (Table 1). Tumours were histologically classified as well differentiated, moderately differentiated or poorly differentiated adenocarcinomas using the WHO criteria [35]. Survival time was calculated from the date of surgery to the date of death or last follow-up with times censored from patients dying of causes unrelated to colorectal cancer and those surviving. Median follow-up was 7 years (from 5 to 9 years).

TABLE 1
UICC classification

UICC stage	TNM stage
Stage 0	$T_{is}\ N_o\ M_o$
Stage I	$T_{1,2}\ N_o\ M_o$
Stage II	$T_{3,4}\ N_o\ M_o$
Stage III	$T\ N_{1,2,3}\ M_o$
Stage IV	$T\ N\ M_1$

Tissue specimens

Sections from the colorectal adenocarcinoma and normal mucosa at the proximal/distal resection margins were obtained at surgical resection. Two pathologists reviewed

the slides of 100 cases. For every case, one paraffin block with both tumour tissue and normal mucosa was selected for the detection of bcl-2, c-erbB-2, p53, ki–67, CD44, laminin and collagen IV expression by immunohistochemistry.

Immunohistochemistry

Five-micrometer thick sections were cut and mounted on glass slides coated wit APS (aminopropylmethoxysilane), dewaxed in xylene and rehydrated with graded alcohols. Endogenous peroxidase was blocked with 3% H_2O_2 for 15 minutes. Before application of the primary antibody, sections were immersed in 10mM citrate buffer (PH = 6.0) and rinsed in TBS (Tris 0.05M, NaCl 0.9%, PH = 7.6) and heated in a microwave oven (650–800W) for three cycles of 5 minutes. In order to reduce non-specific binding, they were washed with TBS buffer and the primary antibodies were placed (Table 2). For bcl-2 staining, we used the bcl-2 antibody (dilution 1:10, Biogenex). For c-erbB-2 the c-erbB-2 antibody (dilution 1:50, DAKO), for p53 the D0–7 antibody (dilution 1:100, Biogenex) against both wild and mutated forms, for ki–67 the MIB–1 antibody (dilution 1:80, DAKO), for CD44 the CD44 antibody clone DF1485 (dilution 1:100, Biogenex), for laminin the laminin antibody (4C7) (dilution 1:300, DAKO) and for collagen IV the collagen IV antibody (dilution 1:100, DAKO). Samples were subsequently in the secondary antibody for 30 minutes and incubated to ABC (avidin-biotin complex) for 30 minutes. Diaminodenzamine (DAB) was used as a chromogene and light hematoxylin counterstain was used. Omission of the primary antibody acted as negative control.

TABLE 2
Antibodies used

Antibodies	Supplier	Dilution
Bcl-2	Biogenex	1:10
c-erbB-2	Dako	1:50
p53(DO–7)	Biogenex	1:100
ki–67(MIB–1)	Dako	1:80
CD44(clone DF1485)	Biogenex	1:100
Laminin(4C7)	Dako	1:300
Collagen IV	Dako	1:100

Evaluation of immunostaining

Immunoreactivity for bcl-2 was evaluated according to the percentage of tumour cells with positive cytoplasmic staining. A cut-off of <5% tumour cells positive was used to define negative cases and >5% positive tumour cells was used to define positive cases. The infiltrating lymphocytes and the neurons were used as positive control. In order to evaluate c-erbB-2 immunostaining a cut-off <25% of neoplastic cells positive was used to define low expression, 25–50% of neoplastic cells positive was used to define moderate expression and a cut-off of >50% of neoplastic cells positive was used to define extensive expression. Immunoreactivity for p53 was evaluated semi quantitatively by two observers and according to the percentage of positive tumour nuclei, scored as follows: low expression, for tumours showing less than 10% immunostained nuclei, moderate expression for tumours showing 10–50% of immunoreactive nuclei, extensive expression for those tumours with nuclear immunoreactivity in more than 50% of tumour cells. For positive controls of p53 expression, we used a known laryngeal carcinoma case with diffuse p53 nuclear accumulation. Immunoreactivity for ki–67 was evaluated according to the percentage of positive tumour cells and scored as follows: 0: for 0% of immunostained tumour cells, +: for 1–10% of immunostained tumour cells, ++ for 11–25%, +++ for 26–50%, ++++ for 51–75% and +++++ for 76–100% immunostained tumour cells. A cut-off of <50% immunostained tumour cells positive was used to define “low” cases and >50% tumour cells positive was used to define “high” cases. As interval positive control, ki–67 positive cells of the normal colonic mucosa were used. They were confined to the middle and lower third of the intestinal crypt. In order to evaluate CD44 immunostaining, tumour cells showing cytoplasmic or membranous staining were regarded as positive. A cut-off <10% of neoplastic cells positive was used to define low expression, 10–50% of neoplastic cells positive was used to define moderate expression and a cut-off >50% of neoplastic cells positive was used to define extensive expression. In order to evaluate laminin immunostaining, the tumours were categorized according to the number of immunopositive tumour cells counted in the sections containing the maximum diameter of tumours as follows: low, less than 20 tumour cells were positive; moderate, 20 to 500 tumour cells were positive; extensive, more than 500 tumour cells were positive. For collagen IV immunostaining, the amount of immunoreactivity at the tumour-stromal border was scored semi-quantitatively. In all cases the overall extent of BM deposition at the tumour cell-stroma interface of the whole section, covering a large tumour area at the site of deepest invasive growth, was scored. More than 75% immunoreactivity at the tumour-stromal interface was scored as extensive BM deposition, between 25% and 75% as moderate, and less than 25% as low. In c-erbB-2, p53, ki–67, CD44, laminin and collagen IV immunostaining, the negative cases were included into the “low” cases group.

Statistical Analysis

All analyses were performed using the statistical packages Minitab 13.0 and S-Plus 3.2. Chi-squared analysis or Fisher's Exact Test as appropriate assessed categorical variables. Continuous data were assessed by Wilcoxon's Rank Sum Test. Kaplan-Meier survival curves were constructed and differences in survival between groups were compared using the Log-Rank Test.

Results

bcl-2

Positive immunohistochemical staining for bcl-2 was seen in 27 of 100 (27%) of the colorectal carcinomas examined. Strong positive staining was seen in infiltrating lymphocytes with in the tumour stroma. Neurones also showed positive. In areas of normal colonic epithelium, bcl-2 expression was seen in the basal cells of crypts. The relationship between bcl-2 expression and a range of clinicopathological variables and the other markers is summarized in Table 3. There was no significant correlation between bcl-2 staining and sex, age, tumour site or tumour grade. However, a statistically significant correlation was detected between bcl-2 staining and tumour stage ($p = 0.012$). From the 27 positive bcl-2 patients, 22 were UICC stage I patients and only 5 were UICC stage III patients. No significant association was demonstrated between bcl-2 and c-erbB-2, p53 and CD44. However, there was very strong evidence of correlation between bcl-2 staining and ki–67, laminin and collagen IV.

Survival Analysis

The patients were followed up for an average of 7 years (from 5 to 9 years). Overall, there was a trend towards increased survival in patients whose tumours expressed bcl-2 protein (Fig. 1). There was also an increased survival in patients whose tumours expressed collagen IV (Fig. 7). On the other hand there was a trend towards decreased survival in patients whose tumours expressed c-erbB-2, p53, CD44 and laminin (Fig. 2, 3, 5, 6). However, there was not significant evidence of a correlation between survival and ki-67 expression (Fig. 4). Correlation of bcl-2 with the other six markers on survival of 100 patients showed an increased survival in patients with positive bcl-2 expression (Fig. 8, 9, 10, 11, 12, 13).

TABLE 3
Clinical and pathological features of 100 patients with colorectal cancer stratified by bcl-2 status

Variable	Immunohistochemical expression of bcl-2		p-value
	Negative bcl-2 (<5%, n = 73)	Positive bcl-2 (>5%, n = 27)	
Sex			
Male (53)	39	14	0.889*
Female (47)	34	13	
Age (years)			
Median	70	69	0.062^
Tumour Stage			
Stage I (61)	39	22	0.012*
Stage III (39)	34	5	
Tumour Site			
Rectum (57)	45	12	
Left Colon (23)	16	7	0.237*
Right Colon (20)	12	8	
Tumour Grade			
Well/Moderate (91)	64	27	0.108◆
Poor (9)	9	0	
Extensive (>50%, n = 39)	31	8	

Patient Survival			
1 year	7	0	
1–3 years	18	4	
3–5 years	38	7	
> 5 years	10	16	
c-erbB-2			
Low (0-25%, n=78)	56	22	
Moderate (25–50%, n=13)	9	4	0.573◆
Extensive (>50%, n=9)	8	1	
p53			
Moderate (10–50%, n = 27)	19	8	0.395*
Extensive (>50%, n = 39)	31	8	
ki–67			
Low (<50%, n = 41)	22	19	0.000*
High (>50%, n = 59)	51	8	
CD44			
Low (0–10%, n = 30)	23	7	
Moderate (10–50%, n = 30)	22	8	0.823*
Extensive (>50%, n = 40)	28	12	
Laminin			
Low (<20, n = 15)	9	6	
Moderate (20–500, n = 59)	39	20	0.003◆
Extensive (>500, n = 26)	25	1	
Collagen IV			
Low (<25%, n = 39)	34	5	
Moderate (25–75%, n = 36)	31	5	0.000*
Extensive (>75%, n = 25)	8	17	

* Chi-Square Test, ^ Kruskal-Wallis Test, ◆ Fisher's Exact Test

Discussion

Tissue growth depends on both cell proliferation and the rate of cell death [27]. Thus, it is conceivable that neoplastic growth may be caused or promoted by factors inhibiting cell death. bcl-2 is a protoncogene that is involved in the regulation of cell death by inhibiting apoptosis in many cell systems in physiologic and neoplastic conditions [27]. The normal biological mechanism of action of bcl-2 is not clear, although the presence of bcl-2 protein is usually associated with favorable clinicopathological features in some neoplasms[16]. The increase of bcl-2 expression in mucosal regeneration following irradiation suggests that activation of bcl-2 may support stem cell survival [34]. The relationship between bcl-2 expression and the evaluation from normal colonic epithelium to invasive cancer is not fully understood. However, there is evidence to

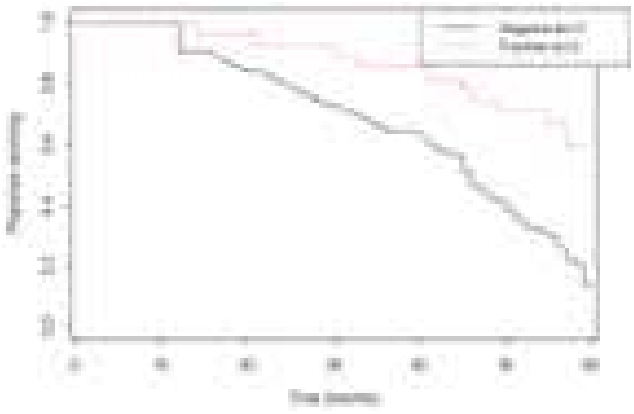

Fig. 1. Survival of 100 patients with colorectal cancer stratified by bcl-2 immunohistochemical staining: bcl-2 (<5%) (negative): 73, bcl-2: (>5%) (positive): 27.

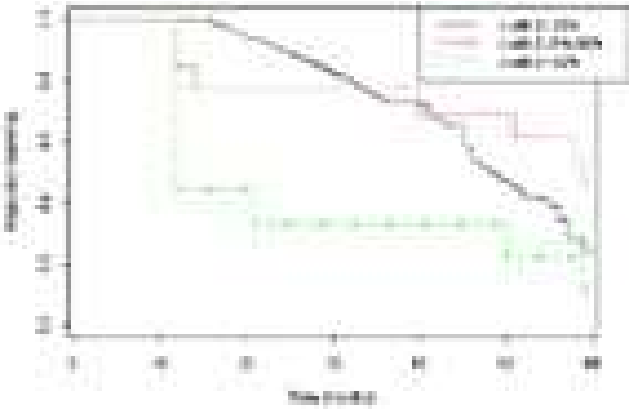

Fig. 2. Survival of 100 patients with colorectal cancer stratified by c-erbB-2 staining: c-erbB-2: <25%, low (n = 78), c-erbB-2: 25–50%, moderate (n = 13), c-erbB-2: >50%, extensive (n = 9).

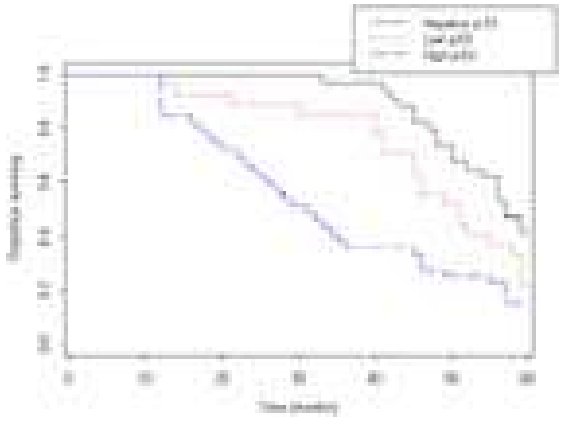

Fig. 3. Survival of 100 patients with colorectal cancer stratified by p53 staining: p53: 0–10%, low (n = 34), p53: 10–50%, moderate (n = 27), p53: >50%, extensive (n = 39).

suggest that bcl-2 expression is lost during the evolution of colorectal cancer [29]. There are different clinical implications of bcl-2 expression in colorectal cancer of various geographical regions. The reason remains unclear, but this

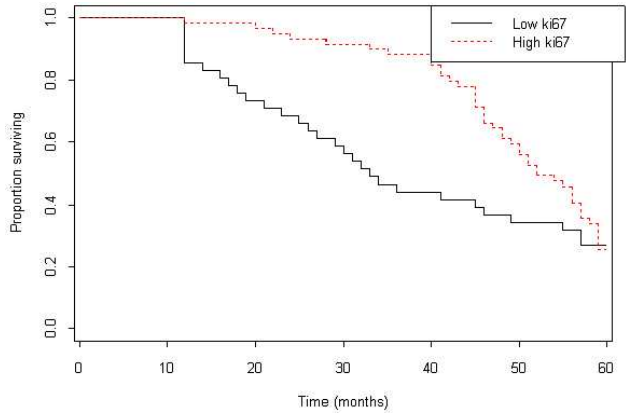

Fig. 4. Survival of 100 patients with colorectal cancer stratified by ki–67 expression: ki–67: <50%, low (n = 41), ki-67: >50%, high (n = 59).

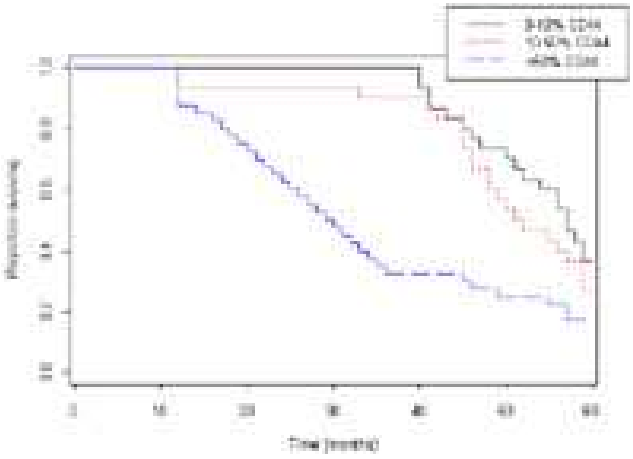

Fig. 5. Survival of 100 patients with colorectal cancer stratified by CD44 staining: CD44: 0–10%, low (n = 30), CD44: 10–50%, moderate (n = 30), CD44: >50%, extensive (n = 40).

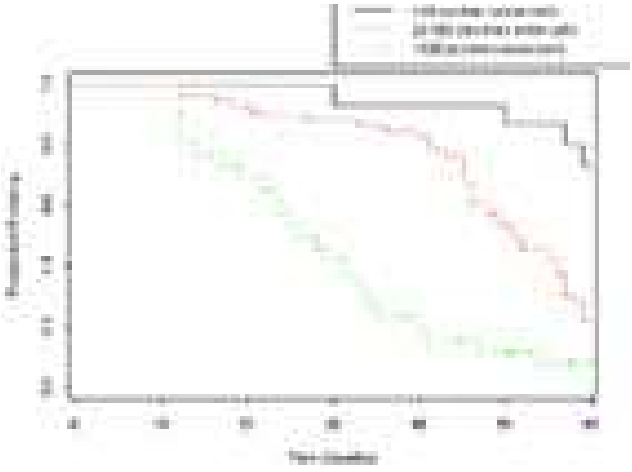

Fig. 6. Survival of 100 patients with colorectal cancer stratified by laminin staining: laminin: <20 positive tumour cells, low (n = 15), laminin: 20–500 positive tumour cells, moderate (n = 59), laminin: >500 positive tumour cells, extensive (n = 26).

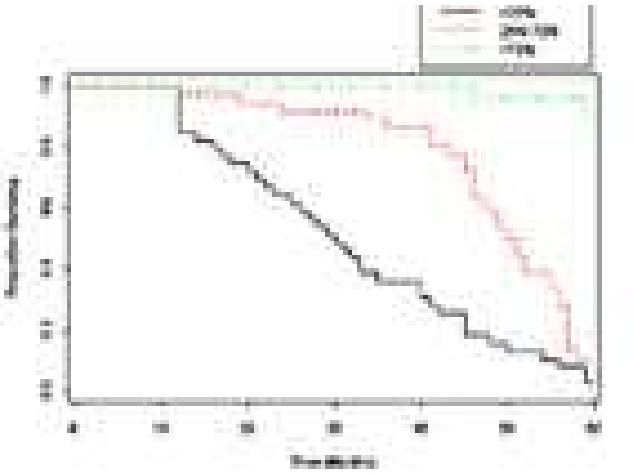

Fig. 7. Survival of 100 patients with colorectal cancer stratified by collagen IV staining: collagen IV: <25%, low (n = 39), collagen IV: 25–75%, moderate (n = 36), collagen IV: >75%, extensive (n = 25).

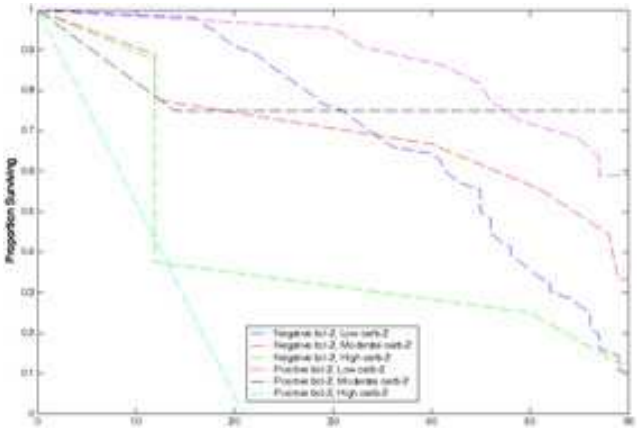

Fig. 8. Correlation of bcl-2 with c-erB-2 on survival of 100 patients.

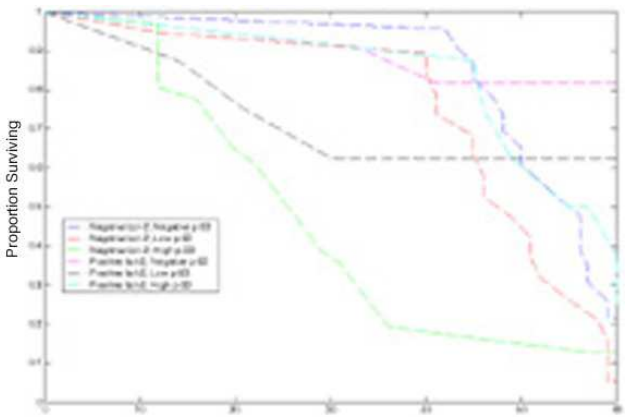

Fig. 9. Correlation of bcl-2 with p53 on survival of 100 patients.

controversy may be related to the characteristics of the patient population on which analyses were performed [22]. The cause of bcl-2 expression in colorectal carcinoma is unclear. One possibility is that the malignant clone is derived from a basal crypt cell and the tumor cell retains bcl-2 expression. Other possibility is translocation of the bcl-2 gene to another chromosomal site in close proximity to powerful enhancer elements in the Ig heavy chain locus, resulting in transcriptional deregulation of the bcl-2 gene and high levels of bcl-2 protein. Other possible mechanisms are mutation of the bcl-2 promoter causing deregulated protein expression and mutation of bcl-2 itself, thereby increasing its half-life. Other possibility is the loss of wild type, functional p53 that could lead to deregulated expression of bcl-2 protein [23]. It has been also suggested that the loss of bcl-2 expression with colorectal tumour progression may be due to wild-type p53 and some p53 mutations

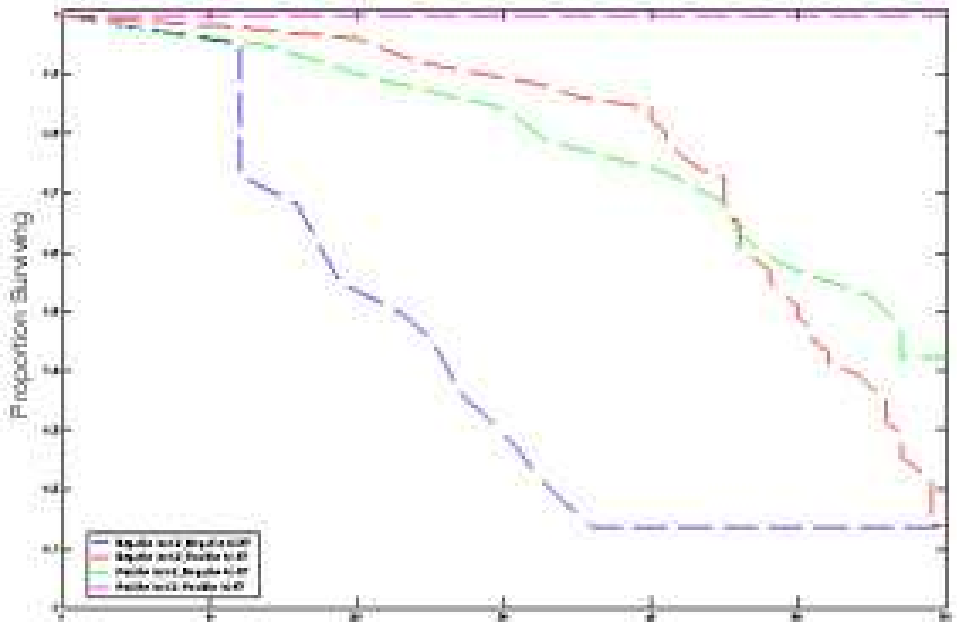

Fig. 10. Correlation of bcl-2 with ki-67 on survival of 100 patients.

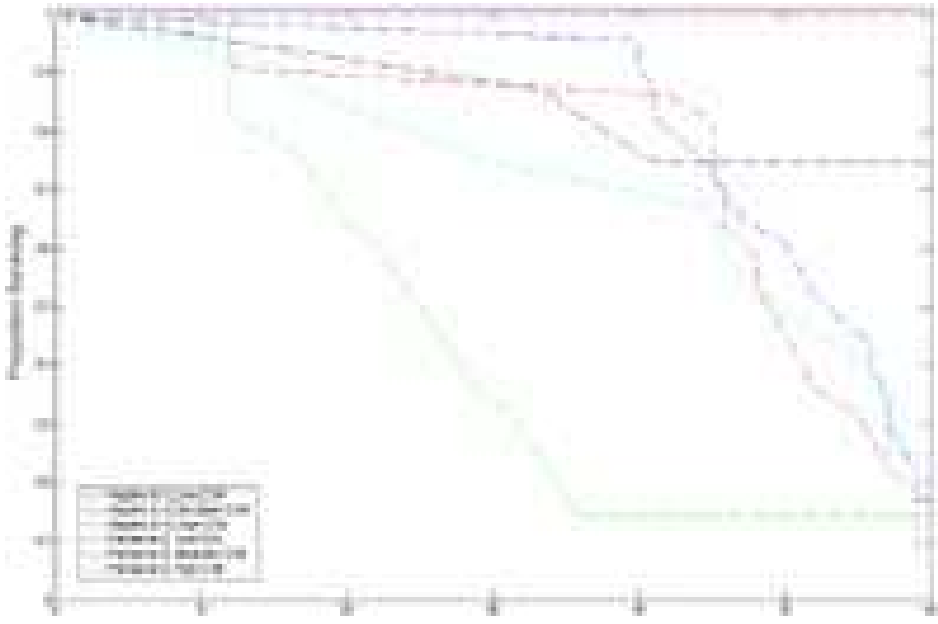

Fig. 11. Correlation of bcl-2 with CD44 on survival of 100 patients.

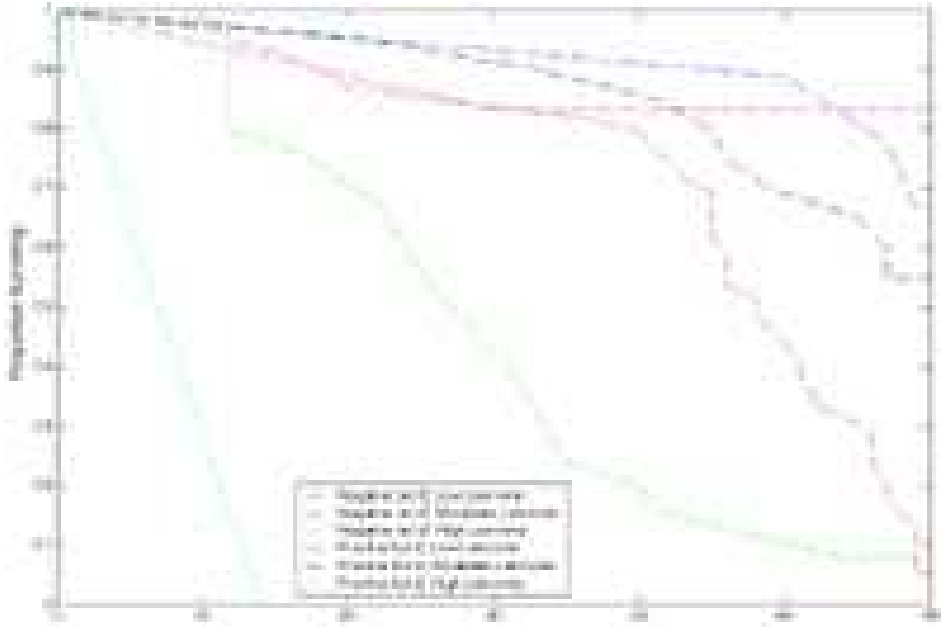

Fig. 12. Correlation of bcl-2 with laminin on survival of 100 patients.

down-regulating bcl-2 by binding to a transcriptional silencer element within the bcl-2 promoter [23].

In our study, bcl-2 immunoreactivity was seen only in the moderately and well differentiated tumours, which is in agreement with previous reports [9, 33]. We found bcl-2 expresssion in 27% of our cases. Our results are somewhat lower than other colorectal cancer studies in which 28–35% of tumours have been positive for bcl-2 [9, 18, 33]. Higher levels of bcl-2 positive cases, ranging from 50% to 67% have also been reported [29, 24]. A statistically significant correlation was detected between bcl-2 staining and tumour stage ($p = 0.012$). Expression of bcl-2 has been also associated with a more favourable clinical outcome in non-small cell carcinoma of the lung and thyroid follicular carcinomas [25]. Bcl-2 expression as reported in breast cancer, was associated with increased survival [21]. In breast cancer, bcl-2 may be more appropriate as an indicator of therapeutic response than a prognostic factor [4]. However, investigations of bcl-2 in colorectal carcinoma have yielded conflicting results in survival. Basari et al and Tollenaar et al did not find any prognostic significance of bcl-2 expression [7, 31]. In contrast, other studies found that bcl-2 expression was associated with a favourable clinical outcome [5, 13]. Many factors should be taken into consideration as an explanation for these conflicting survival results. A difficulty in the interpretation of literature data is the use of different staging systems or small groups of different stages. Furthermore, the details of patient therapy are often inadequate in prognostic

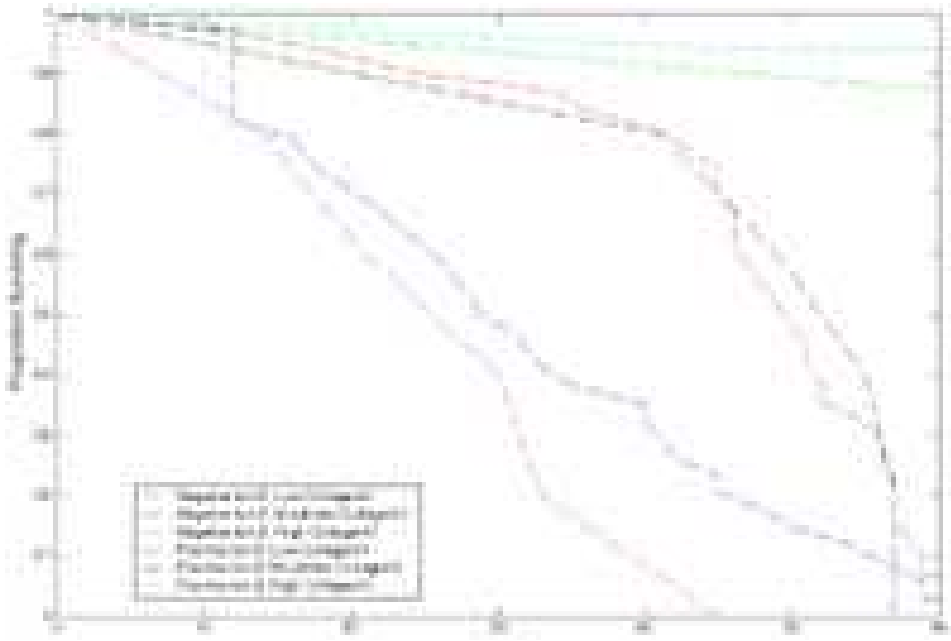

Fig. 13. Correlation of bcl-2 with collagen IV on survival of 100 patients.

marker studies. Several studies include patients with all stages of UICC classification. This kind of studies obviates the need for sub stratification and is, therefore, more appropriate for the assessment of the prognostic utility of a marker. The choice of the patient group is also determined by the questions asked, as markers would have little potential use in stage O comparison to stage I or III disease [8]. Our study with a 5 year follow up, suggests that bcl-2 expression is associated with better clinical course. It seems paradoxical that bcl-2, which inhibits apoptosis, should be associated with good prognosis. However, bcl-2 has also been shown to slow down cell growth in colorectal cancer lines [34]. Loss of bcl-2 may be indicative of a more aggressive phenotype allowing further tumour progression under influences such as p53 changes [18].

C-erbB-2 is one among many genetic factors that has been identified as responsible for the tumourigenesis of colorectal cancer. Several studies suggest that c-erbB-2 protein contributes to tumour cell invasion and metastasis and is significantly correlated with a poor prognosis [38, 39]. Our study has also shown that c-erbB-2 expression in colorectal cancer is associated with poor prognosis. Mutation of p53 is one of the most frequently encountered genetic alterations in solid tumours. p53 mutations play a central role in colorectal tumour progression and are present in >50% of sporadic colorectal carcinomas [17]. In our study there was a statistically significant correlation between survival and p53 expression. p53 expression was associated with poor prognosis. On the other hand we found no association between ki-67 scores and clinical outcome [2]. CD44 is a widely distributed transmembrane glycoprotein that has been proved to function as a major cell-surface receptor for glycosaminoglycan hyaluronate and to play an important role in cell-cell and cell-extracellular matrix interactions such as lymphopoiesis, myelopoiesis, lymphocyte homing, macrophage activation and tumour progression and metastasis [40]. Our study suggests that CD44 expression in colorectal cancer is associated with poor prognosis. Laminin serves as an important adhesion protein for epithelial cells positioned on a basement membrane and plays an important role in cell migration during tumour invasion and tissue remodeling [3]. In our study increased laminin immunoreactivity, suggesting a high invasive potential of tumour cells, was a significant poor prognostic indicator. Basement membranes and the distribution of type IV collagen have been extensively studied in relation to tumour biology and degradation of basement membranes is purported to be a crucial event in the process of tumour invasion and metastatic spread [20]. We found that collagen IV immunoreactivity was an indicator of good prognosis. In order to analyze the joint influences of the seven markers on survival, a correlation between bcl-2 and the other markers has been made. Bcl-2 expression was associated with better prognosis. Apoptosis is a well-organized process controlled by genes with positive or negative regulatory functions [17]. Its role in tumour progression is complex and incompletely understood [41]. Multiple proto-oncogenes, regulatory factors and tumour suppressor genes appear to have a dominant role in the pathogenesis of colorectal cancer. The gene mutations produce metabolically more stable proteins that may be used as prognostic indicators in patients with colorectal cancer [6]. The results from this study would suggest that expression of bcl-2 appear to be useful in selecting a group of colorectal cancer patients with a better prognosis. Immunohistochemical evaluation of bcl-2 in colorectal cancer may be of clinical

value. It may be useful as an adjunctive test in routine histopathological practice.

References

1. *Allegra CJ, Paik S, Colangelo LH et al:* Prognostic value of thymidylate synthase, ki-67, and p53 in patients with Dukes' B and C colon cancer: a national cancer institute-national surgical adjuvant breast and bowel project collaborative study. Journal of Clinical Oncology 2003, 21(2), 241–250.
2. *Allegra CJ, Parr AL, Wold LE et al:* Investigation of the prognostic and predictive value of thymidylate synthase, p53 and ki-67 in patients with locally advanced colon cancer. Journal of Clinical Oncology 2002, 20(7), 1735–1743.
3. *Aoki A, Nakanishi Y, Akimoto S et al:* Prognostic significance of laminin-5 γ2 chain expression in colorectal carcinoma: immunohistochemical analysis of 103 cases. Dis Colon Rectum 2002, 45, 1520–1527.
4. *Barbareschi M, Caffo O, Veronese S et al:* Bcl-2 and p53 expression in node-negative breast carcinoma: a study with long term follow-up. *Human Pathol* 1996, 27, 1149–1155.
5. *Baretton GB, Dielbold J, Christoforis G et al*: Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. *Cancer* 1996, 77, 255–264.
6. *Bhatavdekar JM, Patel DD, Ghosh N et al:* Coexpression of Bcl-2, c-Myc, and p53 oncoproteins as prognostic discriminats in patients with colorectal carcinoma. Dis Colon Rectum 1997, 40, 785–790.
7. *Bosari S, Nonegnini L, Graziani D et al:* Bcl-2 oncoprotein in colorectal hyperplastic polyps, adenomas and adenocarcinomas. Hum Pathol 1995 , 26(5), 534–540.
8. *Bouzourene H, Gervaz P, Cerottini JP et al:* P53 and ki-ras as prognostic factors for Dukes' stage B colorectal cancer. European Journal of Cancer 2000, 36, 1008–1015.
9. *Flohill CC, Janssen PA, Bosman FT:* Expression of bcl-2 protein in hyperplastic polyps, adenomas and carcinomas of the colon. J Pathol 1996, 178, 393–397.
10. *Garrity MM, Burgart LJ, Mahoney MR et al: Prognostic* value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. J Clin Oncol 2004, 22(9), 1572–1582.
11. *Giatromanolaki A, Sivridis E, Stathopoulos GP et al:* Bax protein expression in colorectal cancer. Association with p53, bcl-2 and patterns of relapse. Anticancer Research 2001, 21, 2253–2260.
12. *Haier J, Naxralla M, Nicolson O:* Cell surface molecules and their prognostic values in assessing colorectal carcinomas. Ann Surg 2000, 237, 11–24.
13. *Ilyas M, Hao XP, Wilkinson K et al:* Loss of bcl-2 expression correlates with tumour recurrence in colorectal cancer. Gut 1998, 43, 383–387.
14. *Indinnimeo M, Cicchini C, Giarnieri E, Stazi A, Mingazzini PL, Stipa V:* Evaluation of CD44 variant 6 expression and clinicopathological factors in pulmonary metastases from colon carcinoma. Oncol Rep 2003, 10(6), 1875–1877.
15. *Jeziorska M, Haboubi NY, Schofield PF et al:* Distribution of gelatinase B (MMP-9) and type IV collagen in colorectal carcinoma. Int J Colorect Dis 1994, 9, 141–148.
16. *Joensuu H, Pylkkaanen I, Toikkanen S:* Bcl-2 protein and long-term survival in breast cancer. Am J Pathol 1994, 145, 1191–1198.
17. *Kahlenberg MS, Stoler DL, Rodriguez-Bigas MA et al:* P53 tumour suppressor gene mutations predict decreased survival of patients with sporadic colorectal carcinoma. Cancer 2000, April 15, 88(8), 1814–1819.
18. *Kaklamanis L, Savage A, Whitehouse R et al:* Bcl-2 protein expression: association with p53 and prognosis in colorectal cancer. J Pathol 1998, 77, 1864–1867.
19. *Kouraklis G, Kakisis J, Theoharis S, Tzonou A, Glivanou A, Raftopoulos J, Karatzas G:* Prognostic significance and correlation with survival of bcl-2 and TGF-beta RII in colon cancer. Dig Dis Sci 2003, 48(12), 2284–2289.
20. *Lazaris Ach, Tzoumani AN, Thimara I et al:* Immunohistochemical assessment of basement membrane components in colorectal cancer: prognostic implications. J Exp Clin Cancer Res 2003, 2(4), 599–606.
21. *Manne U, Myers RB, Moron C et al:* Prognostic significance of bcl-2 expression and p53 nuclear accumulation in colorectal adenocarcinoma. Int J Cancer 1997, 74, 346–358.
22. *Manne U, Weiss HL, Grizzle WE:* Bcl-2 expression is associated with improved prognosis in patients with distal colorectal adenocarcinomas. Int J Cancer Pred Oncol 2000, 89, 423–430.
23. *Miyasita T, Harigal M, Hanada M et al:* Identification of a p53 dependent negative response element in the bcl-2 gene. Cancer Res 1994, 54, 3714–3717.
24. *Mueller J, Mueller E, Hoepner I et al:* Expression of bcl-2 and p53 in de novo and ex-addenoma colon carcinoma: a comparative immunohistochemical study. J Pathol 1996, 180, 259-265.
25. *Pilotti S, Collini P, Rilke F et al:* Bcl-2 protein expression in carcinomas originating from the follicular epithelium of the thyroid gland. J Pathol 1994, 172, 337–342.
26. *Pricolo VE, Finkelstein SD, Hansen K, Cole BF, Kirby IB:* Mutated p53 gene is an independent adverse predictor of survival on colon carcinoma. Arch Surg 1997, 132, 371–375.
27. *Reed JC:* Bcl-2 and the regulation of program cell death. J Cell Biol 1994, 124, 1–6.
28. *Reerink O, Karrenbeld A, Plukker JT et al:* Molecular prognostic factors in locally irresectable rectal cancer treated preoperatively by chemo-radiotherapy. Anticancer Res 2004, 24(2c), 1217–1221.
29. *Sinicrope FA, Ruan SB, Cleary KR et al:* Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. Cancer Res 1995, 55, 237–241.
30. *Sun XF, Bartik Z, Zhang H:* Bcl-2 expression is a prognostic factor in the subgroups of patients with colorectal cancer. Int J Oncol 2003, 23(5), 1439–1443.
31. *Tollenaar RA, van Krieken JH, van Slooten HJ et al:* Immunohistochemical detection of p53 and bcl-2 in colorectal carcinoma: no evidence for prognostic significance. Brit J Cancer 1998, 77, 1842–1847.
32. *Visca P, Del Nommo F, Botti C et al:* Role and prognostic significance of CD44s expression in colorectal cancer. Anticancer Res 2002, 22(5), 2671-2675.
33. *Watson AJM, Merrit AJ, Jones LS et al:* Evidence for reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. Br J Cancer 1996, 73, 889–895.
34. *Wilson JW, Nostro MC, Balzi M, Faraoni P, Cianchi F, Becciolini A, Potten CS:* Bcl-w expression in colorectal cancer. Br J Cancer 2000, 82(1), 178–185.
35. *World Health Organization:* International Histological Classifications of Tumours: Histological Typing of Intestinal Tumours. Geneva. WHO, 1976.
36. *Yank HB, Chow NH, Shey BS et al:* The role of bcl-2 in the progression of the colorectal adenoma-carcinoma sequence. Anticancer Research 1999, 19, 727–730.
37. *Yang JL, Hanley JR, Yu Y et al:* In vivo overexpression of c-erB-2 oncoprotein in xenografts of mice implanted with human colon cancer lines. Anticancer Research 1997, 17, 3463–3468.

38. *Yang J-L, Russell PJ, Crowe PJ et al:* Higher expression of oncoprotein c-myc, c-neu, PCNA and p53 in metastasizing colorectal cancer (CRC) than in non-metastasizign colorectal CRC. Ann Surg Onco 1996, 3, 574–579.
39. *Yang J-L, Russell PJ, Crowe PJ et al:* Overexpression of c-erb-2 mRNA and/or c-neu oncoprotein is a predictor for metastases from colorectal cancer. Anticancer Res 1997, 17, 1023–1026.
40. *Zalewski B, Famulski W, Sulkowska M, Sobaniec-Lotowska M, Piotrowski Z, Kisiekewski W, Sulkowski S:* CD44 expression in colorectal cancer: an immunohistochemical study including coorelation with cathepsin D immunoreactivity and some tumour clinicopathological fetures. Folia Histochemica et Cyto Biologica 2001, 39(2), 152–153.
41. *Zavrides H, Zizi-Sermpetzoglou A, Elemenoglou I et al:* Immunohistochemical expression of bcl-2 in Dukes' stage B and C colorectal carcinoma patients: correlation with p53 and ki–67 in evaluating prognostic significance. Pol J Pathol 2005, 56, 4, 179–185.

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