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Prognostic evaluation of CD44 expression in correlation with bcl-2 and p53 in colorectal cancer

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Abstract: To investigate the expression of CD44 in colorectal cancer and examine its association with clinicopathological features, bcl-2, p53 and long-term outcome, paraffin-embedded tumour specimens from 61 patients with Dukes stage B (AJCC/UICC stage I) and 39 patients with Dukes stage C (AJCC/UICC stage III) colorectal adenocarcinoma were assessed by immunohistochemistry. The expression of CD44, bcl-2 and p53 were correlated with 5-year follow-up. Low CD44 expression was present in 30%, moderate in 30% and extensive in 40% of cases. It was not related to patient sex and age but was related to tumour differentiation, stage and tumour site. No association was demonstrated between CD44 and bcl-2. However, there was significant evidence of an association between CD44 and p53 in 66 cases in which p53 was previously assessed. There was a trend towards increased survival in patients whose tumours expressed lower levels of CD44 protein. When entered into multivariate analysis model, which also included bcl-2 and p53, CD44 staining emerged as an indicator of poor prognosis in colorectal cancer patients.

Keywords: CD44 - Colorectal cancer - Bcl-2 - P53 - Prognosis

Introduction

Colorectal cancer results from a series of genetic events, which disorder the normal mechanisms controlling cell growth [15]. Recent studies have identified CD44 glycoproteins as potentially important components of tumour progression and the metastatic cascade [12, 16]. CD44 was originally described as a homing receptor on lymphocytes, mediating lymphocyte interactions with high endothelial venules [18]. Metastasizing tumour cells and recirculating (activated) lymphocytes share several properties including motility and invasive behaviour involving reversible adhesive contacts, accumulation and expansion in draining lymph nodes, adhesion to vascular endothelium and extravasation. This analogy between circulating lymphocytes and tumour dissemination prompted the hypothesis that malignant cells might use molecules like CD44 for metastasizing. Experimental and clinical studies have shown a casual role of specific CD44 isoforms in metastasis formation and documented deregulated CD44 expression in human cancer [29, 35]. On the other hand, contradictory reports concerning the biological role of CD44 in tumourigenesis and its clinical value in prognosis have been also reported [17, 19].

The bcl-2 proto-oncogene is an inhibitor of apoptosis and may therefore permit the accumulation of genetic alterations that influence cell division and potentially contribute to tumour development. The bcl-2 gene is located at chromosome 18q21 and its product is a 24 kD protein localized to the nuclear envelope endoplasmic reticulum and outer mitochondrial membranes [24].

P53 is a tumour suppressor gene that plays a key role in the control of the cell cycle. Cell proliferation is inhibited by normal or wild type 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 cancer and has been associated with adverse postoperative outcome and poor survival [13, 31].

The aim of this study was to investigate CD44 immunoreactivity in colorectal carcinoma and to determine its association with clinicopathological features, bcl-2, p53 and with long-term outcome.

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Materials and methods

Patients. A series of 100 patients underwent surgical resection for primary colorectal adenocarcinoma at the 3rd Department of Surgery, Tzanio Hospital of Athens between 1995 and 1999. Cases of non-inherited polyposis colorectal cancer (NHPCC), familial adenomatus polyposis (FAP) or ulcerative colitis and patients who died in the immediate postoperative period were excluded from this study. None of the patients had received pre- or postoperative radiotherapy or chemotherapy. Each patient was regularly followed up at six-month intervals for a minimum of 5 years. Clinical staging was based on Dukes classification and on clinical evaluation including preoperative chest x-ray, abdominal ultrasonography or computed tomography and abdominal exploration during laparotomy. Tumours were histologically classified as well differentiated, moderately differentiated or poorly differentiated adenocarcinomas using the WHO criteria [36]. 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).

Tissue specimens and immunohistochemistry. Sections from the colorectal adenocarcinoma and normal mucosa at the proximal/distal resection margins were obtained at surgical resection. The slides were reviewed by two pathologists. For every case, one paraffin block with both tumour tissue and normal mucosa was selected for the immunohistochemical detection of CD44, bcl-2 and p53 protein expression.

Five-micrometer thick sections were mounted on glass slides coated with APS (aminopropylmethoxysilane), dewaxed in xylene and rehydrated with graded alcohols. Endogenous peroxidase was blocked with 3% H_2O_2 for 15 min. Before application of the primary antibody, sections were immersed in 10 mM citrate buffer (pH 6.0), rinsed in TBS and heated in a microwave oven (650-800 W) for three cycles of 5 min. In order to reduce nonspecific binding of antisera, sections were washed with TBS before application of the primary antibodies: anti-CD44 (clone DF 1485, dilution 1:100, Biogenex), anti-bcl-2 (dilution 1:10, Biogenex) and anti-p53 (DO-7, dilution 1:100 Biogenex) against both wild and mutated forms. Sections were subsequently treated with the secondary antibody for 30 min and incubated with ABC (avidin-biotin-peroxidase complex) for 30 min. Diaminobenzidine (DAB) was used as a chromogen followed by slight hematoxylin counterstaining. Omission of the primary antibody served as negative control.

In order to evaluate CD44 immunostaining, tumour cells showing cytoplasmic or membraneous staining were regarded as positive. A cut-off <10% of positive neoplastic cells was used to define low expression, 10-50% to define moderate expression and >50% to define extensive expression.

Immunoreactivity for bcl-2 was evaluated according to the percentage of tumour cells with positive cytoplasmic staining. A cut-off <5% of positive tumour cells was used to define negative cases. Strong positive staining was seen in infiltrating lymphocytes within the tumour stroma. The infiltrating lymphocytes and the neurones were used as positive control.

Immunoreactivity for p53 was evaluated semiquantitatively by two observers and according to the percentage of positive tumour nuclei, scored as follows: "negative" for tumours showing <10% of immunostained nuclei, "low" for tumours showing 10-50% of immunoreactive nuclei and "high" for tumours with nuclear immunoreactivity in >50% of tumour cells. For positive controls of p53 expression, we used a known laryngeal carcinoma case with diffuse p53 nuclear immunostaining.

Statistical analysis. All analyses were performed using the Minitab and SPlus statistical packages. Categorical variables were assessed by chi-squared analysis or Fisher's exact test as appropriate. Kaplan-Meier survival curves were constructed and differences in survival between groups were compared using the log-rank test. Multivariate analyses were performed with the Cox proportional hazards model.

Results

Examples of immunostaining are shown in Figures 1-3.

CD44 expression

Low CD44 expression (<10% of neoplastic cells) was present in 30%, moderate (10-50% of neoplastic cells) in 30% and extensive (>50% of neoplastic cells) in 40% of cases. In the normal colorectal mucosa, the cell surface CD44 immunoreactivity was confined to the basal part of the crypts and was expressed in under 10% of crypt cells. There were different staining patterns of CD44 localization: in the normal mucosa, CD44 was expressed in the superficial part of the cell, while in the most carcinomas the staining was localized in the basolateral region of the cell. CD44 expression was found in some lymphocytes and macrophages used as internal controls. Nerve fibres in the submucosa and muscularis externa also showed strong staining of myelin sheaths.

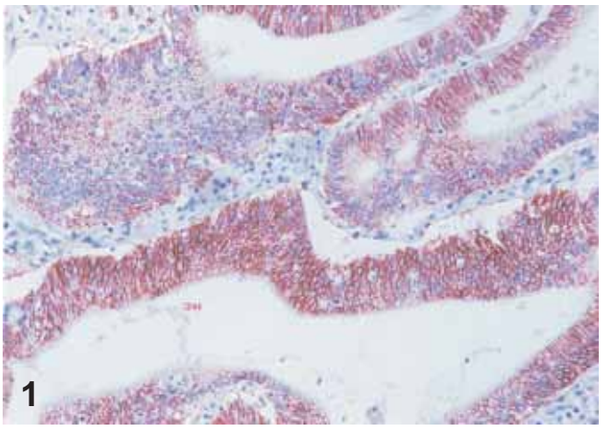
The relationship between CD44 expression and a range of clinicopathological variables is summarised in Table 1. There was a significant correlation between CD44 staining and tumour differentiation (P = 0.0295), as well as tumour stage (P = 0.009) and tumour site (P = 0.000) mainly reflecting the rectal tumours. No significant association was demonstrated between CD44 and bcl-2 status (Tab. 2). However, there was a significant evidence of an association between CD44 and p53 status (P = 0.000), in 66 cases in which p53 had previously been assessed (Tab. 3).

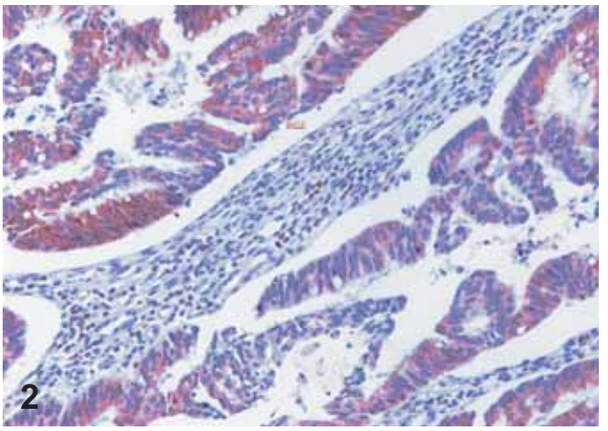
Survival analysis

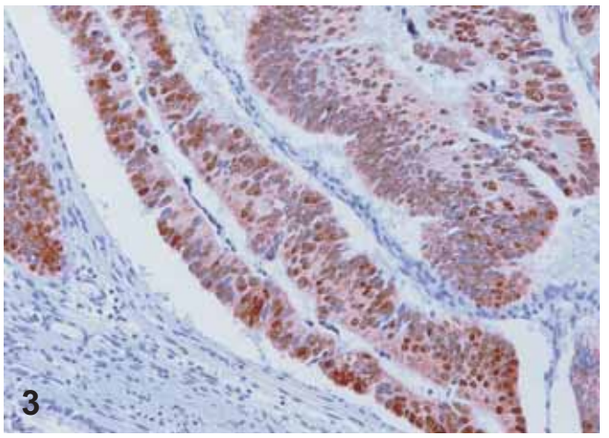
The patients were followed-up for an average of 5 years. Overall, there was a trend towards increased survival in patients whose tumours expressed lower percentages of CD44 protein (P = 0.0004, Fig. 4) and who had tumours expressing bcl-2 protein (P<0.0001, Fig. 5). There was also a statistically significant correlation (P = 0.0002) between survival and p-53 expression (Fig. 6). When entered into a multivariate analysis model (Tab. 4) which also included bcl-2 and p53, CD44 immunostaining emerged as a prognostic indicator variable: patients with >50 % of CD44-positive cells were associated with higher risk of death when compared to patients with <50% CD44-positive cells. In addition, positive bcl-2 expression was related to lower risk of death.

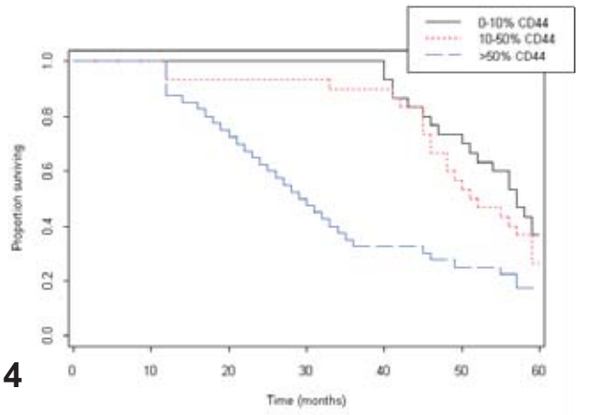
Discussion

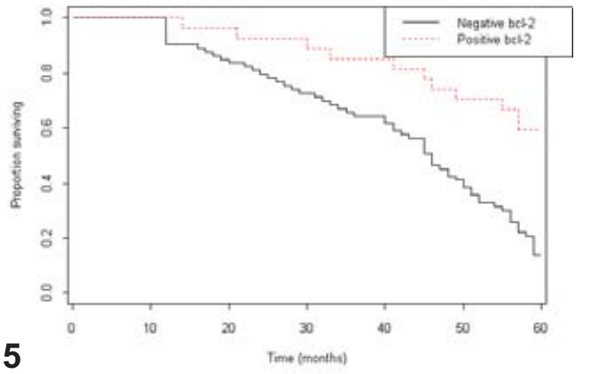
Colorectal cancer is a common disease in Western World and it is one of the most frequent neoplastic diseases in the human population, just after the lung and











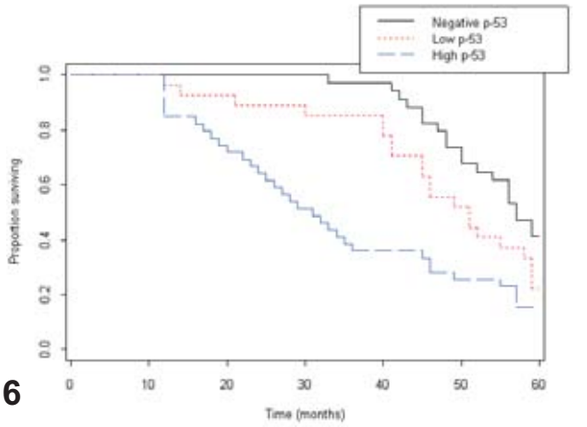


Fig. 1. Immunohistochemical staining of CD44 in colon adenocarcinoma. **Fig. 2.** Adenocarcinoma of the colon with positive immunostaining for bcl-2. **Fig. 3.** Expression of p53 in colon adenocarcinoma. **Fig. 4.** Survival of 100 patients with colorectal cancer stratified by CD44 immunohistochemical staining: <10% (n = 30), 10-50% (n = 30), >50% (n = 40). **Fig. 5.** Survival of 100 patients with colorectal cancer stratified by bcl-2 immunohistochemical staining: bcl-2-negative (<5%) (n = 73), bcl-2-positive (>5%) (n = 27). **Fig. 6.** Survival of 100 patients with colorectal cancer stratified by p53 expression: p53-negative (<10%) (n = 34), low p53 (10-50%) (n = 27), high p53 (>50%) (n = 39).

prostate cancer in men and breast, lung and cervical cancer in women. It evolves through a series of morphologically recognizable stages known as the adenoma-carcinoma sequence. Molecular genetics has increased our understanding of the development of colorectal cancer [22, 28]. Tumour progression is characterized by a stepwise accumulation of specific molecular alterations, ultimately resulting in invasive and metastatic

Table 1. Clinical and pathological features of 100 patients with colorectal cancer stratified by CD44 status

Variable	CD44 immunohistochemistry			
	<10%	10-50%	>50%	P-value
Sex				
Male Female	14 16	14 16	25 15	0.299*
Age (years)				
Median	69.50	68.00	70.50	0.656[a]
Tumour stage				
Stage B Stage C	25 5	14 16	22 18	0.009*
Tumour site				
Rectum Left colon Right colon	28 1 1	22 6 2	7 16 17	0.000[b]
Tumour grade				
Well/moderately differentiated Poorly differentiated	30 0	28 2	33 7	0.0295[b]

*Chi-squared test, [a]Kruskal-Wallis test, [b]Fisher's exact test

Table 2. CD44 expression and bcl-2 status in 100 cases of colorectal cancer

CD44 immunohistochemistry				
bcl-2 (n = 100)	<10%	10-50%	>50%	P-value
Negative (%) (n = 73)	23	22	28	0.823*
Positive (5%) (n = 27)	7	8	12	

*Chi-squared test

Table 3. CD44 expression and p53 status in 66 cases of colorectal cancer

CD44 immunohistochemistry				
P53 (n = 66)	<10%	10-50%	>50%	P-value
Low staining (10-50%) (n = 27)	10	12	5	0.000[b]
High staining (50%) (n = 39)	1	3	35	

[b]Fisher's exact test

cancer. Most of the molecules cause either genetic instability or act on the regulation of the cell cycle, resulting in a disturbed homeostasis between cell proliferation and apoptosis. However, the growth of the primary tumour is not the main cause of the cancer-related death - it is the formation of the metastases in distant organs. A number of studies indicate an important role for CD44 in this complicated process [29, 35, 37].

Table 4. Cox regression model including CD44, bcl-2 and p53

Variable	Coefficient	Standard error	P-value
CD44	0.855	0.289	0.00305
bcl-2	-1.164	0.355	0.00109
p53	0.326	0.316	0.30218

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. This transmembrane glycoprotein molecule is expressed as a standard form (CD44H) and as numerous splice variants (CD44V) [26]. However, the role of CD44 in epithelial metastases is not clear. Some studies suggest that a high level of expression of the variant form of CD44 may be important in tumour invasion, and CD44 has been linked to the development and spread of malignancies [1, 3, 7, 27]. It has been also shown that in lymphomas, as well as in gastric and cervical carcinomas, high CD44 expression is correlated with more advanced tumour stage and possibly with poor prognosis [6, 7]. On the other hand, there are studies in which down-regulation or absence of CD44 indicate aggressiveness [14, 25], or the expression of the CD44 variant epitopes does not correlate with either tumour progression or metastasis to the liver from colorectal carcinoma [11]. It is possible that discrepant results between studies reflect the use of diverse antibodies that may have subtle differences in specificity. The problem is complicated by the existence of numerous CD44 isoforms, which may have remarkable homology in their antigenic repertoire, increasing the possibility of cross-reactivity between the antibodies [9] . Another source of such discrepancies is probably comparison of results obtained with different techniques [8].

Tissue growth depends on both cell proliferation and the rate of cell death [21]. Thus, it is conceivable that neoplastic growth may be caused or promoted by factors inhibiting cell death. Bcl-2 is a proto-oncogene involved in the regulation of cell death by inhibiting apoptosis in many cell systems in physiologic and neoplastic conditions [30]. Investigations of bcl-2 in colorectal carcinoma have yielded conflicting survival results. Kouraklis *et al.* [23] and Tollenaar *et al.* [33] did not find any prognostic significance of bcl-2 expression. In contrast,

other authors reported that bcl-2 expression was associated with a favourable clinical outcome [4, 32].

Mutation of p53 is one of the most frequently encountered genetic alterations in solid tumours. Such mutations play a central role in colorectal tumour progression and are present in 50% of sporadic colorectal carcinomas [20]. Numerous investigations have dealt with the prognostic significance of p53 aberrations in colorectal adenocarcinoma, providing contradictory results [34].

The gene mutations produce metabolically more stable proteins that may be used as prognostic indicators in patients with colorectal cancer [5]. In our study with a 5-year follow-up, expression of CD44 was not related to patient sex and age but was related to tumour differentiation, stage and site. No significant association was demonstrated between CD44 and bcl-2 status. However, there was a significant evidence of an association between CD44 and p53 status in 66 cases in which p53 had previously been assessed. When entered into a multivariate analysis model, which also included bcl-2 and p53, CD44 staining emerged as a prognostic indicator variable.

Both distant metastases and local recurrence after curative operation are the major causes of death in cancer patients. Several studies have been focused on markers to indicate metastatic potential of tumour cells and to predict prognosis. Carcinoma progression is a complex multistep process involving multiple modification of cell surface components, intracellular alterations and genetic changes [2, 10]. CD44 is one of various adhesion molecules. Our study suggests that its expression in colorectal cancer is associated with poor prognosis. Hence, immunohistochemical evaluation of CD44 in colorectal cancer may be of clinical value. Further studies should determine which portion and which function of the CD44 molecule are associated with the metastatic potential of tumour cells.

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