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ORIGINAL ARTICLE

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Glycoprotein CD44 expression in colorectal neoplasms

An immuno-histochemical study including correlation with cathepsin D, extracellular matrix components, p53, Rb, bcl-2, c-erbB-2, EGFR and proliferation indices

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Abstract CD44 has diverse functions in cell–cell and cell–matrix interactions and may be a determinant of metastatic and invasive behaviour in carcinomas. The immunohistochemical expression of CD44 in a series of 110 colorectal carcinomas and 25 adenomas was examined using the monoclonal mouse anti-human phagocytic glycoprotein-1, CD44 (clone DF 1485) in correlation with the expression of basement membrane (BM) antigens (type IV collagen, laminin), fibronectin, cathepsin D, p53, Rb, bcl-2, c-erbB-2, EGFR, proliferation indices (Ki-67, PCNA) and with other conventional clinicopathological variables. In adenomas, low CD44 expression (<10% of neoplastic cells) was present in 16%, moderate (10–50% of neoplastic cells) in 52% and extensive (>50% of neoplastic cells) in 32% of cases. In carcinomas, low CD44 expression was found in 14.5%, moderate in 28.2% and extensive in 57.30%. Although the CD44 expression was higher in carcinomas than in adenomas, we found no statistically significant difference between these two groups. CD44 expression in carcinomas was positively correlated with tumour size (P=0.018), tumour cells cathepsin D (P=0.022), stromal cell cathepsin D (P=0.003) and Rb protein (P=0.021). An inverse correlation was observed between CD44 and the anti-apoptotic protein expression bcl-2 in adenocarcinomas (P=0.039) and in adenomas (P=0.021). These data suggest that CD44 may be involved in the process of invasion and metastasis, probably with the cooperation of cathepsin D. Its expression may be an indicator of poor prognosis in colorectal adenocarcinomas.

Key words CD44 · Cathepsin D · Collagen type IV · Laminin · Fibronectin

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Introduction

CD44 is a transmembrane glycoprotein molecule expressed by many normal tissues and is involved in cell–cell and cell–matrix interactions; it also facilitates lymphocyte recirculation and activation [16, 29]. It is expressed as a standard form (CD44H) and as numerous splice variants (CD44v) [25, 31]. Current evidence suggests that CD44 proteins participate in a large number of related molecular processes, which involve specific adhesions to hyaluronate, collagen, and fibronectin [23], signal transduction [37] and cell migration [34]. CD44 is widely distributed on haematopoietic and nonhaematopoietic cells, including subsets of leucocytes, erythrocytes, many types of epithelium, mesenchymal cells, such as fibroblasts and skeletal muscle cells, glial cells of the central nervous system and a wide variety of tumours [5, 14, 17, 18, 32].

CD44 is normally present in only a few crypt epithelial cells in colorectal mucosa, but expression is markedly increased in carcinomas and their precursor adenomas [12, 38]. CD44 expression has been shown to be associated with metastasis and poor prognosis in colorectal cancer [13, 33, 38]. Some reports link expression in colorectal neoplasms and in some epithelial tissues to cell proliferation, suggesting that CD44 has a role in tumour cell growth [1, 2]. Enhanced expression occurs in a significant fraction of early adenomas before alterations of the K-*ras* protooncogene or p53 tumour suppressor gene [21] and has recently been associated with the expression of mutant p53 protein in colorectal oncogenesis [27]. However, the ability of neoplastic cells to spread to distant sites is derived from complex interrelated processes including the dissolution of basement membrane (BM) and extracellular matrix by degradative enzymes [10, 24, 28, 35]. In colorectal tumours we have examined the immunohistochemical expression of the adhesion molecule CD44 and correlated it with the proteolytic enzyme cathepsin D (CD), BM antigens (collagen type IV and laminin), fibronectin and other potentially valuable prognostic markers (p53, Rb, bcl-2, c-erbB-2, EGFR, prolifera-

tion indices). Conventional clinicopathological features (age, sex, tumour size, tumour grade and Dukes' stage) have also been examined.

Materials and methods

Tissue from surgical specimens resected from 110 patients with primary colorectal carcinoma and from 23 patients with colorectal adenomas was collected and processed by standard techniques to paraffin wax, after fixation in formalin 10% for 24–28 h. In 53 cases one parallel sample was collected shortly after surgical removal and snap-frozen in isopentane liquid nitrogen, using an embedding medium for frozen tissue specimens, O.C.T. Compound (Tissue Tek-Miles). The frozen specimens were stored at –80°C until processing. Consecutive 4-μm sections were stained with haematoxylin and eosin for histological diagnosis. Slides from all the tumours were reviewed and classified according to the World Health Organization (WHO) criteria. In regard to the grade of dysplasia, there were 14 adenomas with low dysplasia and 9 with high dysplasia. Histologically all colorectal carcinomas were adenocarcinomas. The age of patients at the time of surgery ranged from 26 to 86 years. According to WHO criteria, the grade of differentiation was high in 25 cases, moderate in 75 and low in 10 cases. In addition, according to Dukes' stage there were 12 cases of stage A, 55 cases of stage B and 43 cases of stage C disaese. In most cases the adjacent "normal" mucosa was also examined.

For immunohistochemical staining, additional 4-μm-thick sections were cut from paraffin blocks. After blockage of endogenous peroxidase with H_2O_2 in methanol for 30 min, sections were immersed in citrate buffer (pH=6.0) in a microwave-resistant container. The sources and dilutions of the antibodies used are shown in Table 1. Immunoperoxidase detection was employed using the ABC method (Dako) and diaminobenzidine substrate. Counter staining was performed with haematoxylin. Appropriate positive and negative controls were used. For Ki-67 staining cryostat sections 4 μm thick were air-dried and fixed in absolute acetone. They were stained with monoclonal antibodies against Ki-67 (Dako) using sensitive two-step indirect immunoperoxidase technique.

Immunostaining (nuclear or cytoplasmic) was calculated as the percentage of positive tumour cells in relation to the total number in representative fields. Every stained nucleus was considered positive, irrespective of intensity. Only intense membrane immunostaining was considered to represent the overexpression of c-erbB-2 protein, since it has been shown to yield the best prognostic associations [6]. The positivity of cancer cells to cathepsin D (CCCD) was evaluated separately from that of stromal cells (or macrophage-like positive cells) present within or immediately adjacent to the tumour (SCCD). Each sample was first scanned with a low magnification, and at least 10 fields were assessed with a high-power magnification. The results were evaluated quantitatively and divided into several groups (Tables 4, 5). The amount of immunoreactivity of collagen IV and laminin at the tumour–stroma borders was scored semi-quantitatively. More than 10% immunoreactivity at the tumour–stroma interface was scored as extensive BM deposition and less than 10%, as limited. For fibronectin expression we separated the cases into three groups, in accordance with the peripheral tumour staining patterns: an extensive continuous positive connective tissue staining at the periphery of the tumour as (+), positive and negative areas of connective tissue staining as (–/+) and almost negative staining as (–). All slides were reviewed and scored in a blind test by two pathologists.

The association of continuous variables was confirmed using a nonparametric test for two or several independent samples, or Spearman bivariable correlation. *P*-values under 0.05 were considered statistically significant.

Table 1 Antibodies used

Antibodies	Supplier	Dilution	Incubation time
CD44 (Clone DF 1485)	Dako	1:40	Overnight[a]
Cathepsin D (O13 A)	Dako	1: 300	30 min
Type IV Collagen	Dako	1: 100	1 h
Laminin (4C7)	Dako	1: 300	30 min
Fibronectin (A245)	Dako	1: 400	30 min
DO-7 (IgG2b)	Ylem	1: 200	Overnight[α]
anti-BCL-2	Dako	1: 40	Overnight[α]
anti-Rb (AB-5)	Oncogene	1: 80	Overnight[α]
C-erbB-2 (OM-11–925)	ICI, Cambridge	1: 500	1 h
EGFR (AB-4)	Oncogene	1: 500	1 h
PC-10	Dako	1: 50	1 h
Ki-67	Dako	1: 10	30 min
MIB1	Ylem	1: 5	Overnight[α]

[a] With microwave oven antigen retrieval

Results

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. In most adenomas and carcinomas the staining intensity for CD44 was heterogeneous. There were different staining patterns of CD44 localization of the cell surface. In the normal mucosa and some adenomas the CD44 was expressed at the superficial part of the cell, while in most of the carcinomas the staining intensity was localized at the basolateral region of the cell (Fig. 1). CD44 expression was found in some lymphocytes and macrophages used as internal controls. Nerves in the submucosa and muscularis propria also showed strong staining of myelin sheath (Schwann cells). Immunostaining of CD44 was mainly restricted to the superficial part of the adenomas, while in adenocarcinomas there was usually an heterogeneous staining pattern. Immunoreactivity for CD was seen as brown, fine to coarse granular cytoplasmic staining in different patterns of CD localization. In normal mucosa in some adenomas and carcinomas the expression was polarized to the luminal surface in most of the cells, while other cases showed a basal polarization of CD. In adenomas, low CD44 expression (<10% of neoplastic cells) was present in 16%, moderate (10–50% of neoplastic cells) in 52% and extensive (>50% of neoplastic cells) in 32% of the cases. In carcinomas low CD44 expression was found in 14.5%, moderate in 28.2% and extensive in 57.30%. The mean values of the CD44 and the other tumour markers are shown in Table 2. Although the CD44 expression was higher in carcinomas than in adenomas, we did not find any statistically significant difference between these two groups. The CD44 expression with the clinicopathological features and the other potentially prognostic markers are shown in Tables 3–5. CD44 expression was positively correlated with size of tumour (P=0.018), tumour cell cathepsin D (P=0.022), stromal cell cathepsin D (P=0.003) and Rb protein expression (P=0.021) in

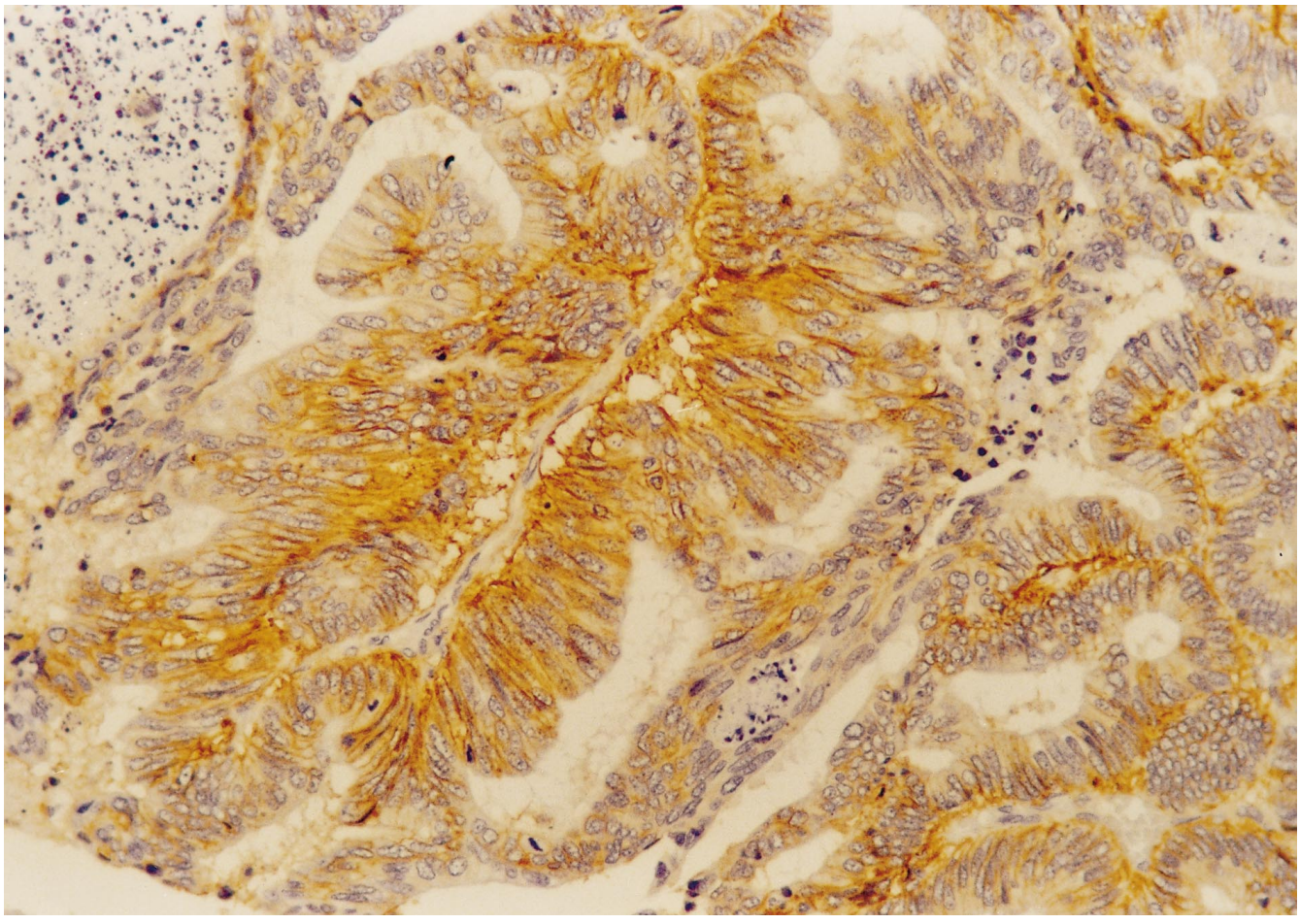

Fig. 1 CD44 positive cells showing cytoplasmic membrane staining localized at the basolateral region in a case of well differentiated colorectal adenocarcinoma. ABC, original magnification ×200

Table 2 Simple statistics

	Mean	SD	Min.	Max.	No.
CD44 Ca	51.01	28.45	0.00	100	110
CD44 adenomas	39.40	29.45	0.00	95	25
CCCD	23.44	23.10	0.00	90	93
p53	35.52	35.44	0.00	95	113
Rb	20.69	23.05	0.00	80	112
bcl-2	4.74	13.87	0.00	75	108
PCNA	67.54	2.74	2	98	110
Ki-67	45.53	16	5.3	82	53

adenocarcinomas. An inverse correlation was observed between CD44 and the anti-apoptotic protein bcl-2 in adenomas (P=0.021) and adenocarcinomas (P=0.039).

Discussion

Loss of adhesive functions and gain of new adhesive functions are thought to play a crucial part in the metastatic cascade in epithelial neoplasms [11], and interactions between tumour cells and the extracellular matrix

Table 3 CD44 expression with clinicopathological features in colorectal cancer

	CD44 expression			*P*-value
	0–10%	10–50%	>50%	
Age				
<50	4	9	21	NS
>50	12	20	39	
Sex				
Female	12	11	30	NS
Male	4	19	31	
Tumour size				
<5	10	15	23	P=0.018
>5	5	11	37	
Grade				
G1	4	7	14	NS
G2	9	22	44	
G3	3	3	4	
Dukes' stage				
A		4	8	NS
B	7	14	34	
C	9	13	21	

Table 4 CD44 expression with potentially prognostic variables in colorectal cancer.

	CD44 expression			*P*-value
	<10	10–50%	>50%	
CCCD				
<10	8	11	18	*P*=0.022
>10	6	11	38	
SCCD				
<10	11	15	23	*P*=0.003
>10	3	7	33	
Collagen IV				
<10	11	17	39	NS
>10	5	10	23	
Fibronectin				
(–)	2	2	3	NS
(–), (+)	1	5	8	
(+)	3	5	10	
p53				
<5	8	15	24	NS
>5	8	16	39	
Rb				
<10	7	17	25	*P*=0.021
>10	9	13	38	
bcl-2				
<5	13	26	48	*P*=0.039
>5	1	4	12	
c-erbB-2				
<25	10	12	27	NS
>25	1	7	5	
EGFR				
<25	15	23	51	NS
>25	1	8	12	
Ki-67				
<50	5	8	12	NS
>50	3	8	14	
PCNA				
<50	4	5	7	NS
>50	11	23	56	

Table 5 CD44 expression with potentially prognostic variables in adenomas

	CD44 expression			*P* value
	<10	10–50%	>50%	
Dysplasia				
Low	2	7	5	NS
High	2	4	3	
CD				
<10	2	1	1	NS
>10	2	9	5	
p53				
<5	4	13	5	NS
>5			1	
Rb				
<5	1	4		NS
>5	3	9	7	
bcl-2				
<5	3	9	5	*P*=0.021
>5	1	4	3	
EGFR				
<25	4	12	7	NS
>25		1		
PCNA				
<50	3	11	3	*P*=0.014
>50		1	4	

(ECM) also play a crucial part in tumour cell spread and metastatic activity. It has been shown that in colorectal carcinomas CD44 proteins homologous to those associated with metastatic potential in animals are overexpressed during tumour progression [12, 38]. 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, 17, 18, 38], but there are studies in which down-regulation or absence of CD44 indicates aggressiveness [12, 22]. It is thus possible that the CD44 expression has differing roles in different parts of the multistep process of carcinogenesis and tumour invasion, perhaps by interaction with extracellular matrix. High levels of CD44 expression could facilitate invasion by migration or by increased adherence to the extracellular matrix. The role of CD44 in the prognosis of human carcinomas is clearly complex and involves modulation of gene expression. CD44 can bind to extracellular matrix molecules such as fibronectin, type IV collagen, laminin and hyaluronate [15, 26], but we found no correlation of CD44 expression with the BM antigens (type IV collagen, laminin) and fibronectin. There was a positive correlation with tumour cell CD, and stromal cell CD, and tumour production of CD, a lysosomal aspartyl protease, has been implicated in tumour invasiveness and metastatic dissemination through the breakdown of extracellular matrix [4]. A correlation of CD44 expression with that of the proteolytic enzyme thus suggests a role for this adhesion molecule in the invasion and metastasis in colorectal tumours. In addition, the expression of CD44 in the superficial part of some adenomas, in which the malignant transformation takes place, may indicate a contribution of this molecule to tumour aggressiveness.

CD44 has recently been associated with mutations of the *p53* gene [13, 27], using Pab 1801 and CM1 antibodies. We found no correlation using the DO7 antigen for immunohistochemical detection of p53 protein. These

conflicting results may have been obtained because different antibodies were used.

The link between CD44 expression in colorectal neoplasms and some epithelial tissues has been correlated with cell proliferation [1, 2]. In colorectal mucosa, CD44 is expressed to a much greater extent in the epithelium of the replicative zone at the base of the colorectal crypt than in the nonproliferating epithelium of the upper crypt and luminal surface. In the reports of other studies [7, 13], we did not find any correlation with the growth fraction of the tumour as estimated by PCNA and Ki-67.

The retinoblastoma gene (*Rb*) product is known to act as a negative regulator of the cell cycle, and lack of pRb expression is considered to be responsible for the genesis of several human malignancies. However, increased expression of pRb has been demonstrated in the majority of colorectal cancers [9], and we found a positive correlation between CD44 and pRb expression. It remains to be seen whether the *Rb* gene modulates the CD44 expression or whether these two molecular markers are independent indicators of poor prognosis in colorectal cancer.

The normal biological mechanism of action of bcl-2 is not clear, although the presence of bcl-2 protein is usually associated with favourable clinicopathological features in some neoplasms [8, 19]. The relationship between bcl-2 expression and the evolution from normal colonic epithelium to invasive cancer is not fully understood. However, there is evidence to suggest that bcl-2 expression is lost during the evolution of colorectal cancer [30], and it has been shown that there is an inverse relationship between bcl-2 and p53 expression in cells of both colorectal adenomas and carcinomas [36]. In this study we found an inverse relationship between CD44 and bcl-2 protein expression in both adenomas and carcinomas. In keeping with other studies [3, 20, 30, 36], this fact suggests that bcl-2 expression is characteristic of the early phase of colorectal carcinogenesis, while enhanced expression of CD44 occurs relatively late in the carcinogenetic process.

This study shows that CD44 expression is positively correlated with cathepsin D and pRb, and inversely with bcl-2 protein. These markers might be of clinical value in colorectal cancer and may help to predict the biological behaviour of the tumour.

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