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Comparison of CD44 expression in early colorectal carcinomas of the de novo and *ex adenoma* types

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Abstract Small colorectal carcinomas without morphological evidence of origin from an adenoma have been called "de novo" carcinomas. As changes in the expression of the adhesion molecule CD44 and its variants have been described along the adenoma-carcinoma sequence in colorectal carcinoma, we compared patterns of CD44 expression in early de novo and ex-adenoma colorectal carcinomas by staining specimens from a group of early (pT1) colorectal carcinomas by immunohistochemistry for CD44 (standard and variant forms v3, v5, v6, v7, v7/8, v10). We evaluated carcinoma, adenoma (ex-adenoma cases), transitional mucosal areas and apparently nonneoplastic mucosa peripheral to the lesions (when present). A marked increase was seen in numbers and intensity of standard and variant forms of CD44 in carcinomatous areas compared with nonneoplastic mucosa in both groups, with no significant difference between the groups. However, adenoma areas of the ex-adenoma cases and the transitional mucosa of the de novo carcinomas had nearly identical staining patterns. Together with data from other molecular studies, this may be interpreted as evidence for an adenoma-type precursor lesion in so-called de novo colorectal carcinomas.

Key words CD44 · Colorectal carcinoma · Carcinogenesis · Immunohistochemistry

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Introduction

CD44, first identified in lymphocytes in 1982 [36], is a transmembrane molecule found to be present on a wide variety of cell types, including those in epithelia, and the tumours derived from them. CD44 is a particularly interesting molecule, since, although it is encoded for by only one gene on chromosome 11, it is expressed in several variant forms owing to both posttranslational glycosylation and differential splicing of various exons [30, 35] which add additional extracellular domains to the molecule. In keeping with its wide distribution and complex expression pattern, various functions have been identified for CD44 including cell adhesion, cell–matrix interactions, signal transduction, and cell migration. Possibly analogous with their function in lymphocyte cell migration, CD44 variant molecules have been shown to confer metastatic capability in rat tumour cell lines [8]. Homologues of these variants have been found in several human malignancies, including non-Hodgkin's lymphomas [18], breast [32], and the stomach [11], where they have been reported to be associated with tumour progression and a worse prognosis [17, 20, 33].

Within the colon, CD44 is normally only weakly expressed, as the "s" or standard form in just a few cells at the bases of crypts [15]. Variant forms of CD44 have been found to be expressed in inflammatory conditions, such as ulcerative colitis [28], and in neoplasms, including adenomas and carcinomas [12]. A progression in the numbers of variant forms (in particular v5 and v6) [26], the number of cells that express these forms, and the intensity of expression has been correlated with the so-called adenoma–carcinoma sequence, with v5 appearing relatively early in the adenoma stage and v6 appearing later in carcinomas as a correlate of invasive and metastatic potential. The expression of CD44v6 has been said to be associated with a poor prognosis in colon carcinoma [25], and testing for CD44v6 has been proposed as screening method for colon carcinoma [6].

Although the majority of colon carcinomas are thought to arise via the adenoma–carcinoma sequence,

which has been well characterized both morphologically [23] and by molecular biology [37], there are reports in the literature [19, 31], primarily in Japan but also increasingly in Europe and America, of colon carcinomas with no histological evidence of origin from an adenoma. Such carcinomas have been called “de novo” carcinomas, since morphologically they seem to arise de novo from normal colon mucosa. From a biological point of view, however, the concept that these carcinomas might develop without a precursor lesion is difficult, if not impossible, to accept and has led to a general lack of acceptance of their existence in the Western world [5].

The clinical importance of these lesions lies in the fact that they become invasive at a small size without forming a conspicuous polypoid lesion. For this reason, they have probably been overlooked in the past [22], but with improvements in endoscopy and better appreciation of their existence, they have been increasingly described [14, 19]. Although there is no universally accepted definition of de novo colorectal carcinoma, several studies [3], including our own [24], have defined them as carcinomas with a diameter of 1 cm or less with submucosal invasion and without elements of adenoma in their vicinity. Clinicopathological studies have indicated that they behave more aggressively than the usual ex-adenoma carcinomas, since they show a higher frequency of lymph node metastases than do ex-adenoma carcinomas of the same stage [2, 34].

Few molecular biological studies of de novo colorectal carcinomas exist, but one of these, our own study of p53 and bcl-2 protein expression [24], has shown a phenotypic profile that fits in with the clinicopathological impression that they are more aggressive than ex-adenoma carcinomas of the same stage. Since certain splice variants of CD44 have been said to be associated with an increased metastatic potential, and since the pattern of expression of the various splice variants of CD44 has been described for the adenoma–carcinoma sequence, we undertook the present comparative study, with groups of pT1 de novo and ex-adenoma carcinomas, using a panel of nine antibodies for CD44 and its variants to see

Table 1 Clinicopathological data for the carcinomas

No.	Sex	Age	Specimen	Location	Size (cm)	G	pT	pN	M	L[b]
De novo carcinomas										
1	F	80	Polypectomy[a]	Sigmoid	0.8	2	1	1	0	–
2	M	59	Polypectomy	Sigmoid	0.7	1	1	×	×	–
3	M	63	Polypectomy	Rectum	0.5	2	1	×	×	+
4	M	82	Polypectomy	Sigmoid	0.7	2	1	×	×	+
5	F	73	Polypectomy	Sigmoid	1.0	3	1	×	×	–
6	M	71	Polypectomy	Sigmoid	0.6	2	1	×	×	–
7	M	55	Partial resection	Ascending	1.0	2	1	1	0	–
8	F	57	Polypectomy	Sigmoid	1.0	2	1	×	×	–
9	M	62	Polypectomy	Sigmoid	0.8	2	1	×	×	–
10	F	75	Polypectomy	Rectum	0.9	2	1	×	×	–
11	M	67	Polypectomy	Sigmoid	0.9	2	1	×	×	–
12	M	83	Polypectomy[a]	Rectum	0.7	2	1	1	0	+
13	F	51	Polypectomy[a]	Rectum	1.0	3	1	0	0	+
14	F	68	Polypectomy	Sigmoid	1.0	2	1	×	×	+
15	M	54	Polypectomy	Sigmoid	1.0	2	1	×	×	+
16	M	51	Polypectomy	Sigmoid	0.6	2	1	×	×	–
17	M	73	Polypectomy	Sigmoid	0.6	3	1	×	×	+
18	F	70	Polypectomy	Descending	0.7	2	1	×	×	+
Ex-adenoma carcinomas										
1	M	62	Polypectomy	Sigmoid	0.4	1	1	×	×	–
2	F	67	Polypectomy	Sigmoid	1.7	2	1	×	×	–
3	M	70	Polypectomy	Sigmoid	2.0	2	1	×	×	+
4	F	55	Polypectomy	Rectum	2.0	2	1	×	×	–
5	F	68	Polypectomy	Sigmoid	2.0	1	1	×	×	–
6	F	67	Polypectomy	Right flexure	1.9	2	1	×	×	–
7	F	65	Polypectomy	Rectum	2.0	2	1	×	×	–
8	F	70	Polypectomy	Rectum	1.9	2	1	×	×	+
9	F	54	Polypectomy	Rectum	2.0	1	1	×	×	–
10	F	74	Polypectomy	Sigmoid	1.9	2	1	×	×	–
11	F	79	Polypectomy	Rectum	1.0	2	1	×	×	–
12	F	77	Polypectomy	Rectum	1.5	2	1	×	×	–
13	M	42	Polypectomy	Sigmoid	2.0	1	1	×	×	–
14	M	81	Polypectomy	Sigmoid	3.2	2	1	×	×	+
15	M	69	Polypectomy	Rectum	3.0	1	1	×	×	–
16	F	86	Polypectomy	Sigmoid	1.9	2	1	×	×	–
17	M	57	Polypectomy	Sigmoid	0.8	2	1	×	×	–
18	F	57	Polypectomy	Sigmoid	1.9	2	1	×	×	–
19	M	64	Polypectomy[c]	Sigmoid	1.2	2	1	0	0	+

[a] Followed by partial resection
[b] Lymph vessel invasion

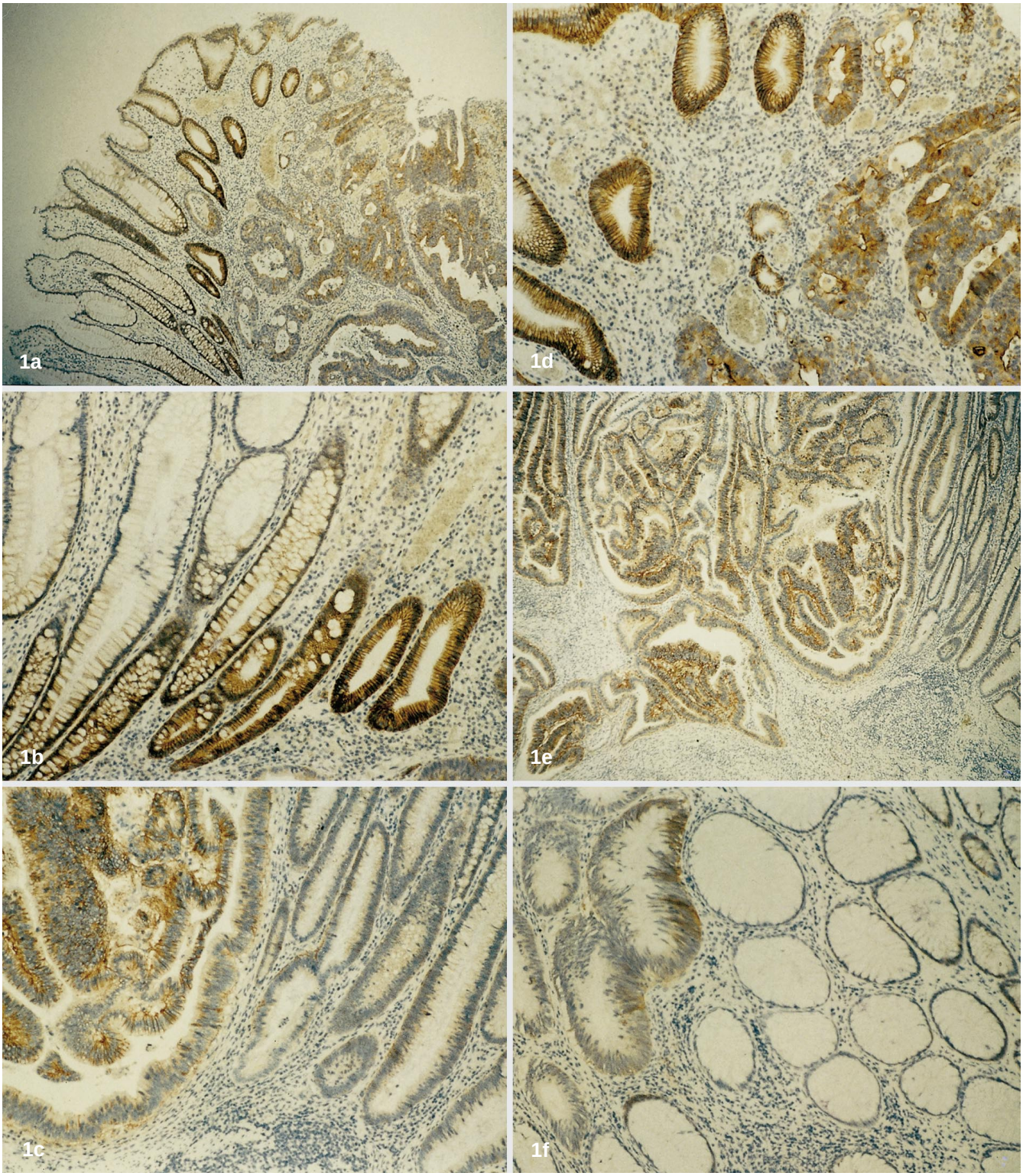


Fig. 1a–f The immunohistochemical reaction for CD44v6 (antibody VFF18) in a de novo (**a–c**) and an ex-adenoma (**d–f**) carcinoma. The carcinomatous areas of both tumour types show a strong reaction. The transitional mucosa of the de novo and the adenomatous areas of the ex-adenoma carcinoma also show a reaction. The normal appearing mucosa of both types show some reaction in the crypt bases only (**c** left-hand side, **f** right-hand side). Original magnifications **a** ×40, **b** ×160, **c** ×160, **d** ×40, **e** ×160, **f** ×160

whether the expression pattern of CD44 in the two tumour types differed. In particular, we wished to see whether the expression of CD44v6 was markedly stronger in the de novo carcinoma group, indicating a greater metastatic potential, and whether the expression of the various variants in different portions of the lesions (carcinoma, adenoma, transitional and nonneoplastic mucosa) could provide any clues as to the origin of the so-called de novo colorectal carcinoma.

Materials and methods

The 37 cases for study consisted of paraffin-embedded tissue from 18 de novo and 19 ex-adenoma colorectal carcinoma specimens. Most of the lesions were removed by polypectomy, and for some of the lesions additional pathological data were available from a subsequent partial resection. For this study, a de novo carcinoma was defined as a carcinoma of 1.0 cm or less in diameter (to minimize the possibility of tumour destruction of a pre-existent adenoma) with definite invasion of the submucosa but not beyond (pT1) and without any evidence of adenoma (low-grade dysplasia) in the adjacent mucosa. The ex-adenoma cases also had invasion of, but not beyond, the submucosa (pT1) and had definite adenoma elements (low-grade dysplasia) in the adjacent mucosa. The tumours in the de novo group ranged from 0.5 to 1.0 cm in diameter, with a mean size of 0.8 cm. The polyps in the ex-adenoma group were 1.81 cm in average diameter, with a range of 0.4–3.2 cm. The carcinomas in the ex-adenoma group were not measured, but each was smaller than the polyp that contained it.

The clinical data of the patients and TNM classification of the lesions in the study are shown in Table 1.

All antibodies for immunohistochemistry were generated by immunizing BALB/c mice with a glutathione *S*-transferase fusion protein containing the human variant CD44 portion v3–v10. The exon-specificity of the antibodies was determined by ELISA and Western blotting against defined CD44 fusion proteins and synthetic peptides as described previously [18]. The following antibodies were used: SFF304 (pan-CD44; 5 µg/ml), VFF327 (CD44v3; 5 µg/ml), VFF8 (CD44v5; 500 ng/ml), VFF4 (CD44v6; 1 µg/ml), VFF7 (CD44v6; 1 µg/ml), VFF18 (CD44v6; 250 ng/ml), VFF9 (CD44v7; 5 µg/ml), VFF17 (CD44v7/8; 10 µg/ml), and VFF16 (CD44v10; 10 µg/ml). The optimal antibody concentration for each antibody was determined by staining of skin keratinocytes. The three CD44v6-specific antibodies recognize different but overlapping epitopes within exon v6, with VFF18 showing the highest and VFF7 the lowest antibody affinities [13].

Prior to incubation with the primary antibody, paraffin sections (2–3 µm) were deparaffinized in Rotihistol (Roth, Germany) three times for 10 min each and then rehydrated in alcohol in a declining series of concentrations. The sections were then heated in a microwave oven in an antigen retrieval buffer (DAKO, Denmark) according to the manufacturer's instructions. Staining was performed in a DAKO TechMate 500 staining system using the biotin-avidin-peroxidase detection system (DAKO ChemMate). A negative control was performed with each of the cases (using an isotype-matched mouse monoclonal antibody instead of the primary antibody), and a skin biopsy sample was performed with each antibody as a positive control.

The staining reactions were evaluated independently by two observers. Each case was evaluated in a maximum of four areas; carcinoma (present in all cases), adenoma (present in all ex-adenoma cases), the transitional mucosa directly adjacent (within 3–4 crypts) to carcinoma or adenoma and nonneoplastic mucosa on the periphery of the section. The reaction was scored semiquantitatively as to its intensity (negative, weak, moderate or strong), its pattern (heterogeneous or homogenous) its frequency (percentage of cells staining) and its distribution (superficial vs deep portion of the crypt). At least 10% of cells with a definite reaction in a given area were required to classify an area as positive.

Results

In general, an increase in the numbers of CD44 variants expressed and their intensity could be seen in both the ex-adenoma and the de novo groups from peripheral mucosa with a normal appearance towards the carcinoma (Fig. 1, 5). The most frequently expressed forms were CD44v6 (antibody VFF18) (Fig. 1), pan-CD44 (antibody SFF304) (Fig. 3), CD44v5 (Fig. 2) and CD44v6 (antibodies VFF4 and VFF7), while variants v7/8, v7 and v10 were only expressed in a few cases in the two groups. CD44v3 showed weak to moderate expression in only a very small number of ex-adenoma cases. The overall trend in terms of numbers of cases and strength of expression of CD44 and its variants was not markedly different between the two groups. In both groups there was an increase in carcinomatous tissue compared with normal for pan-CD44, CD44v5 and CD44v6 (see Fig. 5), only a minor increase for CD44v7/8 and CD44v7, and no noticeable increase for CD44v10 and CD44v3.

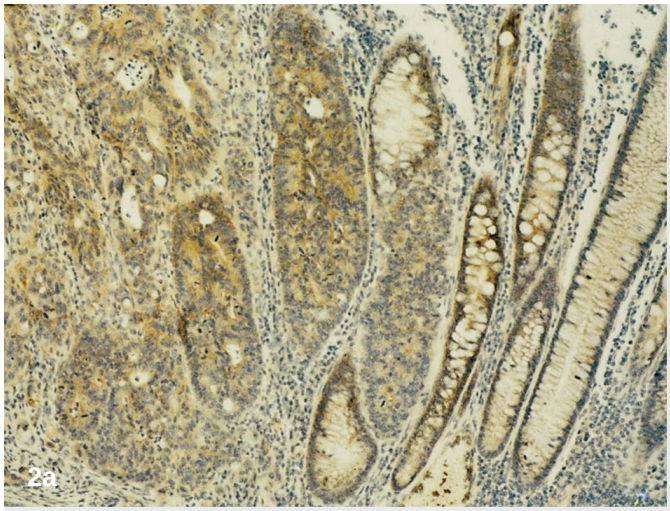
Evaluation of the pattern of staining with the various antibodies revealed that the mucosa with a normal appearance had homogeneous staining which was seen in the crypt bases in the peripheral apparently normal mucosa (Fig. 1) and in the full thickness of the transitional mucosa adjacent (within 3–4 crypts) to the carcinomatous or adenomatous areas of the lesions. In both adenomas and (in particular) in carcinomas, the pattern of staining was quite heterogeneous (Fig. 3). No correlation was found between grade, lymph node status or presence

Fig. 2a, b The immunohistochemical reaction for CD44v5 (antibody VFF8) in **a** de novo and **b** ex-adenoma carcinomas. The reaction in the de novo carcinoma is strong in the adjacent transitional mucosa and moderate in the carcinoma. The reaction in the ex-adenoma carcinoma is weak in the adenoma (right-hand side) and stronger in the carcinomatous areas (left-hand side). Original madnifications **a** ×80, **b** ×160 ▶

Fig. 3 The immunohistochemical reaction for pan-CD44 (antibody SFF304) in a de novo carcinoma demonstrating marked heterogeneity in the carcinoma with the strength of reaction varying from negative to strongly positive. Original magnification ×160

Fig. 4 A case of de novo carcinoma with immunohistochemistry for VFF18 (CD44v6). The reaction in the transitional mucosa adjacent to the carcinoma is strong while the reaction in the carcinoma is only weakly positive in focal areas. Original magnification ×80

Fig. 5 Comparison of CD44 expression in the de novo and ex-adenoma carcinomas by histological area. The immunohistochemical reactions for pan-CD44 and variants v3, v5, v6, v7, v7/8 and v10 in the de novo and ex-adenoma carcinoma groups. The various antibodies are shown for each histological area along the *x*-axis (*s* pan-CD44 antibody SFF304, *3* CD44v3 antibody VFF327, *5* CD44v5 antibody VFF8, *6a* CD44v6 antibody VFF7, *6b* CD44v6 antibody VFF4, *6c* CD44v6 antibody VFF18, *7* CD44v7 antibody VFF9, *7/8* CD44v7 and CD44v8 antibody VFF17, *10* CD44v10 antibody VFF16), and the number of cases for each area along the *y*-axis. A progression in the numbers of CD44 variants and the intensities of staining is evident for both groups. Similarities between the carcinoma areas of the two groups and the adenoma of the ex-adenoma and the transitional mucosa of the de novo group are also seen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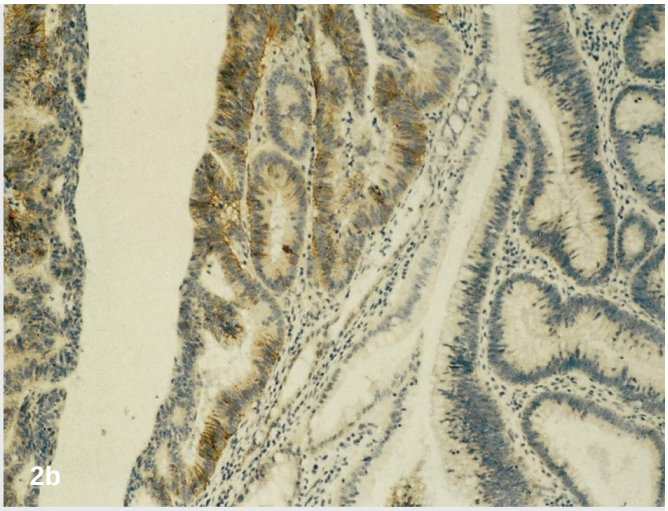
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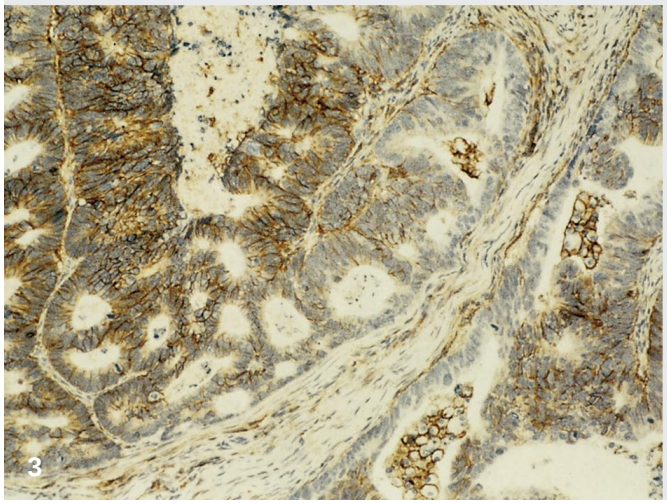
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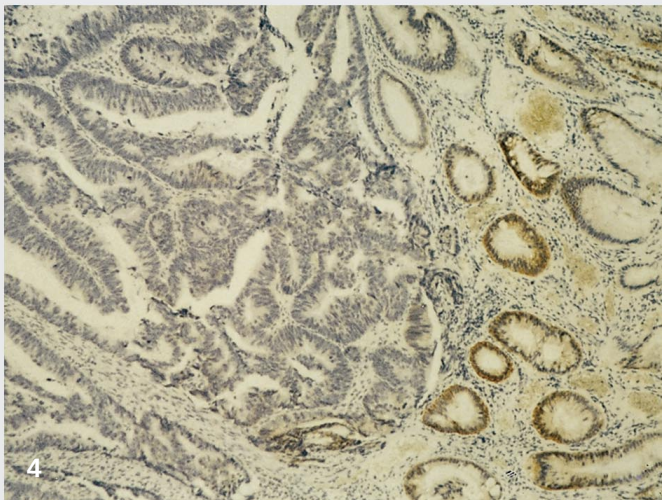
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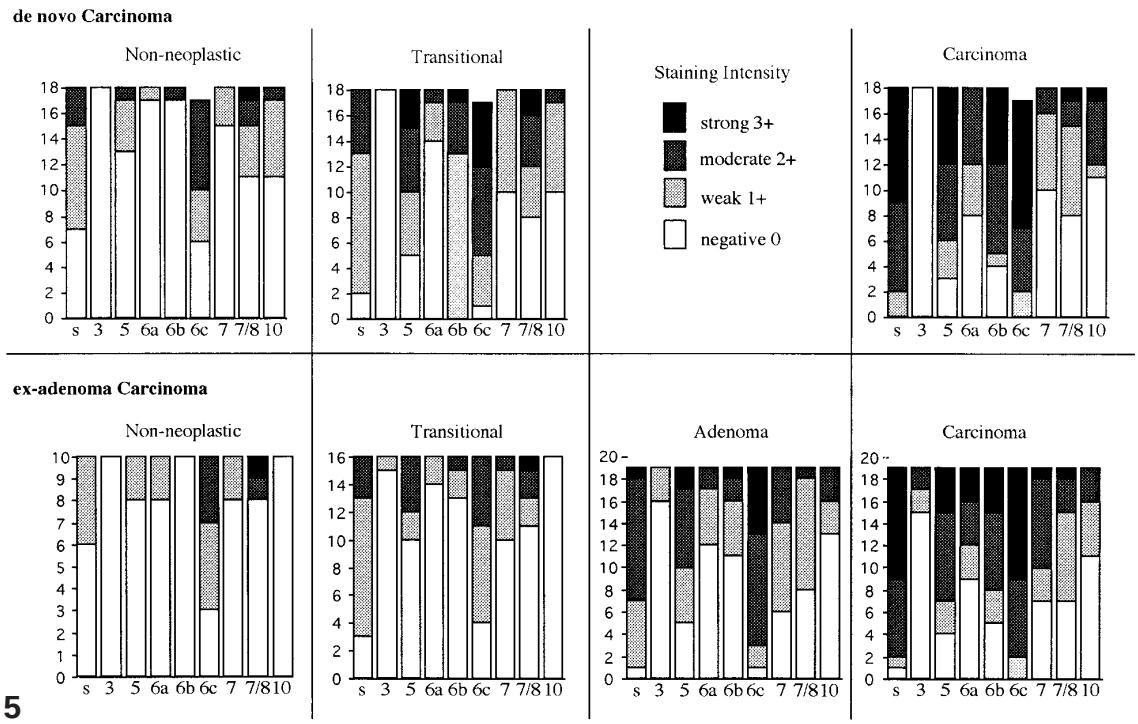
de novo Carcinoma
Non-neoplastic
Transitional
Staining Intensity
strong 3+
moderate 2+
weak 1+
negative 0
Carcinoma
ex-adenoma Carcinoma
Non-neoplastic
Transitional
Adenoma
Carcinoma
s 3 5 6a 6b 6c 7 7/8 10

5

of lymph vessel invasion and the expression of CD44 and its variants.

When the different areas of the cases in the two groups were compared the carcinomatous areas of both groups were found to show strong expression of pan-CD44, CD44v5 and CD44v6 (VFF18) with no observable differences between the de novo and ex-adenoma types. The adenomatous areas of the ex-adenoma group and the transitional mucosa of the de novo group showed a very similar pattern of expression (Fig. 5). The numbers of cases and the intensity of staining of these two areas (pan-CD44, CD44v5 and CD44v6) was nearly identical, while the transitional mucosa of the ex-adenoma group had much less expression than this area in the de novo group. A few cases in the de novo group even had a paradoxically higher expression of CD44v6 (VFF18) in apparently normal mucosa than in the directly adjacent carcinoma (Fig. 4).

Also striking was the difference in staining reactions with the three antibodies for CD44v6; VFF18, VFF4 and VFF7. VFF18 showed a frequency and intensity of staining that were even higher than those seen with SFF304, while VFF4 staining was somewhat less pronounced than CD44v5 staining and VFF7 showed staining in only a few cases, and it was only of moderate intensity. These differences in staining intensities are also reflected by the in vitro properties of the three antibodies, where VFF18 shows the highest affinity for CD44v6 [13]. Contrary to a previous report [15], this high-affinity antibody showed definite, strong staining for CD44v6 in the apparently normal mucosa of several cases (Figs. 1, 5). No correlation was seen between staining for CD44v6 and lymph vessel invasion identified on H&E-stained tissue sections.

Discussion

This study is the first to describe the pattern of expression of CD44 and its variants in a group of de novo colorectal carcinomas in comparison with a group of ex-adenoma colorectal carcinomas of the same early stage (pT1). Previous studies [26, 38] of CD44 expression in the colon have tried to correlate tumour development and progression along the adenoma–carcinoma sequence by studying the expression of pan-CD44 and, primarily, the variant forms CD44v5 and CD44v6 in samples of apparently normal epithelium, adenomas of various stages and carcinomas. The general pattern that has emerged is that CD44 s is expressed relatively early along this sequence, then CD44v5 in adenomas, and then CD44v6 in adenomas with high-grade dysplasia and in carcinoma. Although our study is not directly comparable with these earlier investigations, we saw a similar topographical progression of expression of CD44v5 and CD44v6 from peripheral mucosa with a normal appearance to adjacent transitional mucosa and then to adenoma and carcinoma. An exception to this pattern was seen in our result for CD44v6 using the antibody VFF18, in that more frequent reactivity was seen than for either SFF304 (pan-CD44) or VFF8 (CD44v5). This finding could be explained by the fact that VFF18 has a higher affinity for its epitope than do CD44v6-specific antibodies, which were generally used in previous studies, and is therefore able to detect even a low level of CD44v6 expression [13, 29].

The first goal of this study was to see whether de novo carcinomas, which have been reported to have a higher rate of lymph node metastases than typical ex-adenoma carcinomas of the same stage [34], had a stronger or more frequent rate of expression of CD44v6, which has been correlated with metastatic potential in cell lines [8] and colon carcinoma [25, 38]. This was not the case. Comparison of both the carcinomatous areas and the apparently nonneoplastic mucosa distant from the carcinomas or adenomas showed the same pattern and intensity of CD44 staining (both pan-CD44 and variant forms, including CD44v6) in both groups. In addition, we saw no correlation between CD44v6 staining and lymph vessel invasion in either group.

The second goal of this study was to see whether the expression pattern of CD44 and its variants could provide some information about the possible mechanism of origin of the de novo carcinoma. In particular, we were looking for any observable difference between the two groups that might point to an alternate carcinogenetic mechanism for de novo carcinoma. In this regard, an interesting feature emerges when the patterns of CD44 expression in the different histological areas of the two groups are compared as shown in Fig. 5. A progressive increase in the numbers and intensity of expression of CD44 and its variants is seen in both the ex-adenoma and de novo groups. The transitional mucosa of the ex-adenoma group shows a slightly increased expression of CD44, a finding that has been described previously [7] and has generally been thought to be a secondary effect of tumour, similar to other morphological and immunohistochemical changes in transitional mucosa, which have been described extensively [4, 9, 16]. Unexpectedly, however, the transitional mucosa of the de novo group showed a high expression of CD44, which was nearly identical to that in the adenoma areas in the ex-adenoma group. The most likely explanation for such a similarity is that the “transitional” mucosa of the de novo lesions, which appears to be normal or reactive in routine histology, represents remnants of an adenoma or adenoma-like precursor lesion. To date, few studies have investigated molecular genetic abnormalities in de novo carcinomas, but the available data indicate that the same genetic loci as are important for the adenoma–carcinoma sequence, such as p53 and adenomatous polyposis coli (APC), are also involved in the carcinogenesis of these lesions [1, 10]. It has been hypothesized that the so-called flat adenoma of the colon is the likely precursor of de novo carcinoma [27], with the essential difference from the “conventional” adenoma–carcinoma sequence being that malignant transformation occurs at a much smaller size, without a significant polypoid growth component. Our own data, showing a significantly higher rate of p53 pro-

tein overexpression in de novo versus ex-adenoma carcinomas [24], support this idea. The finding that both flat adenomas and de novo carcinomas have very low rates of *K-ras* mutation compared with polypoid adenomas and ex-adenoma carcinomas [21] may be a clue to the underlying carcinogenetic mechanism, in which key mutations of genes such as *p53* occur much earlier than normal and result in malignant transformation, before other mutations, such as *K-ras*, which are important for polypoid growth, have occurred.

The results of the present study point to a similarity in the carcinogenetic pathways of de novo and ex-adenoma colorectal carcinoma with regard to CD44 expression and suggest the existence of an adenoma-like precursor lesion in the development of so-called de novo carcinoma. As a consequence, from a biological point of view, the term "de novo", which is based on the lesion's histomorphology, is inappropriate. However, it is important to separate de novo carcinomas from the usual colorectal carcinoma developing from polypoid adenomas since they are more difficult to detect endoscopically and must be carefully sought. They have also undergone malignant transformation at a much smaller size than usual. CD44 expression cannot explain this early transformation, but ongoing comparative molecular genetic studies of de novo versus ex-adenoma carcinomas may provide important clues.

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