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Detection of Tn, sialosyl-Tn and T antigens in hereditary nonpolyposis colorectal cancer

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Abstract The simple mucin-type carbohydrate antigens Tn, sialosyl-Tn, T and the 'cryptic' sialylated variant of the last represent the mucin core oligosaccharide structures that are produced in the initial steps of the mucin biosynthetic pathway. Utilizing monoclonal antibodies anti-Tn antigen (HB-Tn1), anti-sialosyl-Tn antigen (HB-STn1), anti-T antigen (HB-T1) and the biotinylated *Amaranthus caudatus* agglutinin (ACA), we have investigated the expression of the simple mucin-type carbohydrate antigens in hereditary nonpolyposis colorectal cancer (HNPCC; 15 cases) compared with sporadic colorectal cancer (CRC; 60 cases) and normal colonic mucosa (30 cases). A variable positivity of Tn, sialosyl-Tn, T and the cryptic sialylated form of this latter antigen was encountered in both HNPCC and sporadic CRC cases; in addition, in normal colonic mucosa a constant reactivity was encountered only for Tn and the cryptic sialylated form of T, while negative results were always obtained for sialosyl-Tn and T antigens. Statistical analysis, performed using a Chi-square test, showed significantly lower ($P = 0.037$) expression of sialosyl-Tn and higher ($P = 0.022$) expression of T in HNPCC than in sporadic CRC, suggesting a greater presence of β1,3 galactosyltransferase activity in HNPCC than in sporadic CRC. We were unable to identify a peculiar phenotype for HNPCC with simultaneous evaluation of reactivity for HB-Tn1, HB-STn1, HB-T1 and ACA; the biological significance of the preferential expression of T antigen in HNPCC remains to be investigated.

Key words Colorectal cancer · Hereditary nonpolyposis colorectal cancer · Carbohydrate antigens · Lectin histochemistry · Immunohistochemistry

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Introduction

Mucins are high-molecular-weight glycoproteins consisting of a central polypeptidic structure (apomucin) to which numerous carbohydrate chains are attached in bottle-brush fashion by an *O*-glycosidic linkage [4, 26, 27]. These oligosaccharide side chains vary in length, although the first step in their biosynthetic pathway is always represented by linkage of *N*-acetyl-D-galactosamine (GalNAc) to serine (ser) or threonine (thr) residues of apomucin, forming the structure named Tn antigen (GalNAcα1→O-ser/thr) [33, 35]. The Tn antigen acts as a precursor for the synthesis of other simple mucin-type carbohydrate antigens; in fact, the subsequent addition of sialic acid gives rise to sialosyl-Tn antigen (NeuAcα2→6GalNAcα1→*O*-ser/thr), whereas if D-galactose is added the T antigen (Galβ1→3GalNAcα1→*O*-ser/thr) is formed [14]. This latter, also known as Thomsen-Friedenreich antigen, may be further sialylated, transforming it to its 'cryptic' form [5, 32]. All these antigenic structures are known as the core region of mucin oligosaccharides [11]. In normal glandular epithelial tissues the expression of simple mucin-type carbohydrate antigens is generally masked by sequential addition of sugars under the control of specific glycosyltransferases that give rise to different oligosaccharide chains [11]; nevertheless, the synthesis of the sialosyl-Tn antigen acts as a 'stop', preventing further chain elongation [11, 16, 17].

Aberrant and incomplete glycosylation is a cancer-associated phenomenon that may determine the truncated synthesis of the oligosaccharide component of mucin and the exposure of core structure [6, 11, 42]. The presence of simple mucin-type carbohydrate antigens has been studied with various lectins and antibodies in several human neoplasms, including bladder cancer [22], carcinoma of the uterine endometrium and cervix [8, 9], ovarian tumours [10, 36], breast cancer [34], carcinoma of the salivary glands [5, 38], pancreatic tumours [13, 25] and gastric carcinoma [3].

The expression of Tn, sialosyl-Tn, T and the cryptic sialylated form of T antigens has also been studied in be-

nign and malignant neoplasms of the large bowel [1, 2, 11, 12, 14, 16–20, 23, 24, 32, 37, 41–43]; nevertheless, insufficient data are reported about their expression in hereditary nonpolyposis colorectal cancer (HNPCC), which represent approximately 4–10% of total colorectal cancer (CRC) cases [1, 21, 28–30, 40].

In the present study, using monoclonal antibodies and the lectin amaranthin (ACA), we have investigated the expression of Tn, sialosyl-Tn, T and the cryptic sialylated form of this last antigen in HNPCC to evaluate their expression compared with that in sporadic CRC.

Table 1 Clinico-pathological data in HNPCC and sporadic CRC (*HNPCC* hereditary nonpolyposis colorectal cancer, *CRC* colorectal cancer)

	HNPCC (n = 15)	Sporadic CRC (n = 60)
Sex		
Male	10	32
Female	5	28
Age (mean age)	43–74 (57.7)	41–90 (68)
Tumour site		
Right hemicolon	4	21
Left hemicolon	7	21
Rectum	4	18
Histological type		
Adenocarcinoma	9	18
Mucinous carcinoma	6	12
Histological grading		
Grade 1	1	18
Grade 2	7	25
Grade 3	1	5
Stage		
Stage I	3	9
Stage II	5	21
Stage III	5	18
Stage IV	2	12

Materials and methods

A total of 75 colorectal carcinomatous surgical samples obtained from the same number of patients were included in this study: 15 patients exhibited the "Amsterdam criteria" for HNPCC syndrome [21, 39], while 60 patients were affected by sporadic CRC. This latter group consisted of patients without evidence of HNPCC, familial adenomatous polyposis or idiopathic inflammatory bowel disease, while preoperative radiation and chemotherapy were excluded for all cases. Twenty biopsies from the right and left colon of each of 10 patients affected by irritable bowel syndrome and 10 surgical specimens from right and left colon of 5 patients in whom resection was performed for megacolon or traumatic lesions were also utilized as normal control tissues. All specimens were fixed in 10% neutral-buffered formalin for 12–24 h at room temperature (RT) and embedded in paraffin at 56°C. From each block serial 4-μm-thick sections were obtained. The grade and stage of carcinomas were determined according to the criteria of Jass and Sobin [15] and Hermanek and Sobin [7], respectively. The main clinicopathological data of the 75 cases of HNPCC and sporadic CRC are summarized in Table 1.

The antigens investigated, structures of carbohydrate epitopes and reagents used for their detection are given in Table 2. Staining was performed by the avidin–biotin peroxidase complex (ABC; Vector Laboratories, Burlingame, Calif.). All slides were incubated with 0.3% H_2O_2 in absolute methanol for 30 min at RT to block the endogenous peroxidase activity, and then washed with phosphate-buffered saline (PBS), pH 7.4. One slide for each case studied was successively treated with 10% ovalbumin (Sigma Chemical Co., St. Louis, Mo., lot no. 68F8150) in PBS for 30 min at RT, rinsed in PBS, and incubated overnight with the biotinylated ACA (working dilution 10 μg/ml in PBS) at 4°C. Additional sections were first treated with normal nonimmune serum for 30 min at RT and then incubated overnight with mouse monoclonal antibodies (MAbs) HB-Tn1 (w.d. 1:200), HB-STn1 (w.d. 1:200), and HB-T1 (w.d. 1:200) at 4°C. The sections incubated with MAbs were also treated with biotinylated goat anti-mouse IgG for 30 min at RT. Finally, ABC complex for 30 min at RT and 3,3′ diaminobenzidine tetrahydrochloride-H_2O_2 substrate solution for 10 min at RT in darkness, were applied. All slides were slightly counterstained with Mayer's haematoxylin.

All series included positive controls; for negative controls the step of incubation with the primary antibodies or lectin was omitted.

Immunostained sections were estimated by light microscopy using ×20 and ×40 objective lenses and a ×10 eyepiece. Slides were independently evaluated by two pathologists without knowledge of the final diagnosis. For each sample the reactivity for all antibodies or lectin utilized was categorized with respect to the location in the supranuclear region (Golgi area), diffuse in the cytoplasm, at the luminal cell membrane, in goblet cell vacuoles and in glandular secretion; in addition, the intensity of immunoreaction

Table 2 Antigens, structures of carbohydrate epitopes and reagents used for their detection

Antigen	Epitope structure	Reagent	Source
Tn	GalNAcα1→O-ser/thr	HB-Tn1 (MAb)	Dako, Copenhagen, Denmark (M 896; lot no.072)
Sialosyl-Tn	NeuAcα2→6GalNAcα1→O-ser/thr	HB-STn1 (MAb)	Dako, Copenhagen, Denmark (M 899; lot no.062)
T	Galβ1→3GalNAcα1→O-ser/thr	HB-T1 (Mab)	Dako, Copenhagen, Denmark (M 898; lot no.072)
		ACA (lectin)	Vector Laboratories, Burlingame, Calif. (lot no.A0405)
Cryptic T[a]	NeuAcα2→3Galβ1→3GalNAcα1→O-ser/thr	ACA (lectin)	Vector Laboratories, Burlingame, Calif. (lot no.A0405)

[a] To identify the cryptic sialylated form of T antigen combination of positive reaction for ACA and negative staining with HB-T1 was utilized

was subjectively evaluated as weak, moderate or strong. A similar sytem to that reported by Therkildsen et al. [37] was used to score the staining reactions for semiquantitative purposes: 1, a few scattered positive cells or scant contents of mucin (<5%); 2, well-defined areas with positive staining of cells or mucin (5–50%); 3, large areas with positive staining of cells or mucin (>50%). The higher value was recorded when a sample showed a difference between the immunoreactive area of tumour cells and mucin. In general, a good correlation (92.6% of the cases) and reproducibility was obtained by the two observers; in the cases where evaluation differed, consensus was achieved after discussion.

Statistical analysis was performed using a Chi-square test with Yate's correction.

Results

Semiquantitative results regarding reactivity for HB-Tn1, HB-STn1, HB-T1 and ACA in normal colonic mucosa, HNPCC and sporadic CRC are reported in Table 3.

For HB-Tn1 in normal colonic mucosa, irrespective of whether it was from the left or the right side of the colon, a weak to moderate immunoreactivity was found in all samples. This was mainly localized in the supranuclear region of the cells of the crypts and on the surface epithelium; only 2 cases exhibited a moderate positivity diffuse in the cytoplasm. No staining was encountered in goblet cell vacuoles.

In HNPCC and in sporadic CRC specimens, diffuse positivity in the cytoplasm of neoplastic elements was observed in 100% of cases (Fig. 1a), while immunoreactivity localized at the luminal cell membrane was encountered in 13.3% of HNPCC and 10% of sporadic CRC (Fig. 1a); in addition, positivity of the glandular secretion was present in 40% and 20% of HNPCC and sporadic CRC cases, respectively (Fig. 1a, b). Immunoexpression, in both neoplastic conditions, was heterogeneous with moderately to strongly immunoreactive neoplastic cells intermingled with negative ones.

The normal colonic mucosa was unreactive for HB-STn1 in all cases.

In HNPCC and sporadic CRC immunoreactivity was present in 73.3% and 95% of cases, respectively; the difference between these two groups was statistically significant ($\chi^2 = 4.343$; $P = 0.037$). In reactive cases, a heterogeneous pattern was observed with a moderate to strong staining intensity localized in the cytoplasm (HNPCC: 90.9%; sporadic CRC: 100%), at the luminal cell membrane (HNPCC: 54.5%; sporadic CRC: 63.2%), in the glandular secretion (HNPCC: 63.6%; sporadic CRC: 78.9%) and within vacuoles of neoplastic elements that exhibited goblet cell differentiation (HNPCC: 27.2%; sporadic CRC: 26.3%) (Fig. 2a, b). In addition, some hereditary and sporadic mucinous carcinomas showed strong positivity of intraglandular mucus, together with a negative or weak reactivity of some neoplastic cells (Fig. 2c).

In normal colonic mucosa negative results were always obtained for HB-T1.

In both HNPCC and in sporadic CRC immunoreactivity was present (in 60% and in 25% of cases, respective-

Table 3 Detection of HB-Tn1, HB-STn1, HB-T1 and ACA in normal colonic mucosa, HNPCC and sporadic CRC with staining score of reactive cases (*HB-Tn1* monoclonal antibody anti-Tn antigen, *HB-STn1* monoclonal antibody anti-STn antigen, *HB-T1* monoclonal antibody anti-T antigen, *ACA Amaranthus caudatus agglutinin*)

	HB-Tn1				HB-STn1				HB-T1				ACA			
	Positive rate	Staining score			Positive rate	Staining score			Positive rate	Staining score			Positive rate	Staining score		
		1	2	3		1	2	3		1	2	3		1	2	3
Normal mucosa	100% (30/30)	3.3%	3.3%	93.3%	0	0	0	0	0	0	0	0	100% (30/30)	0	23.3%	76.7%
HNPCC	100% (15/15)	26.7%	40%	33.3%	73.3% (11/15)	45.4%	27.3%	27.3%	60% (9/15)	33.3%	66.7%	0	100% (15/15)	0	0	100%
Sporadic CRC	100% (60/60)	10%	15%	75%	95% (57/60)	15.8%	57.9%	26.3%	25% (15/60)	40%	60%	0	90% (54/60)	11.1%	38.9%	50%

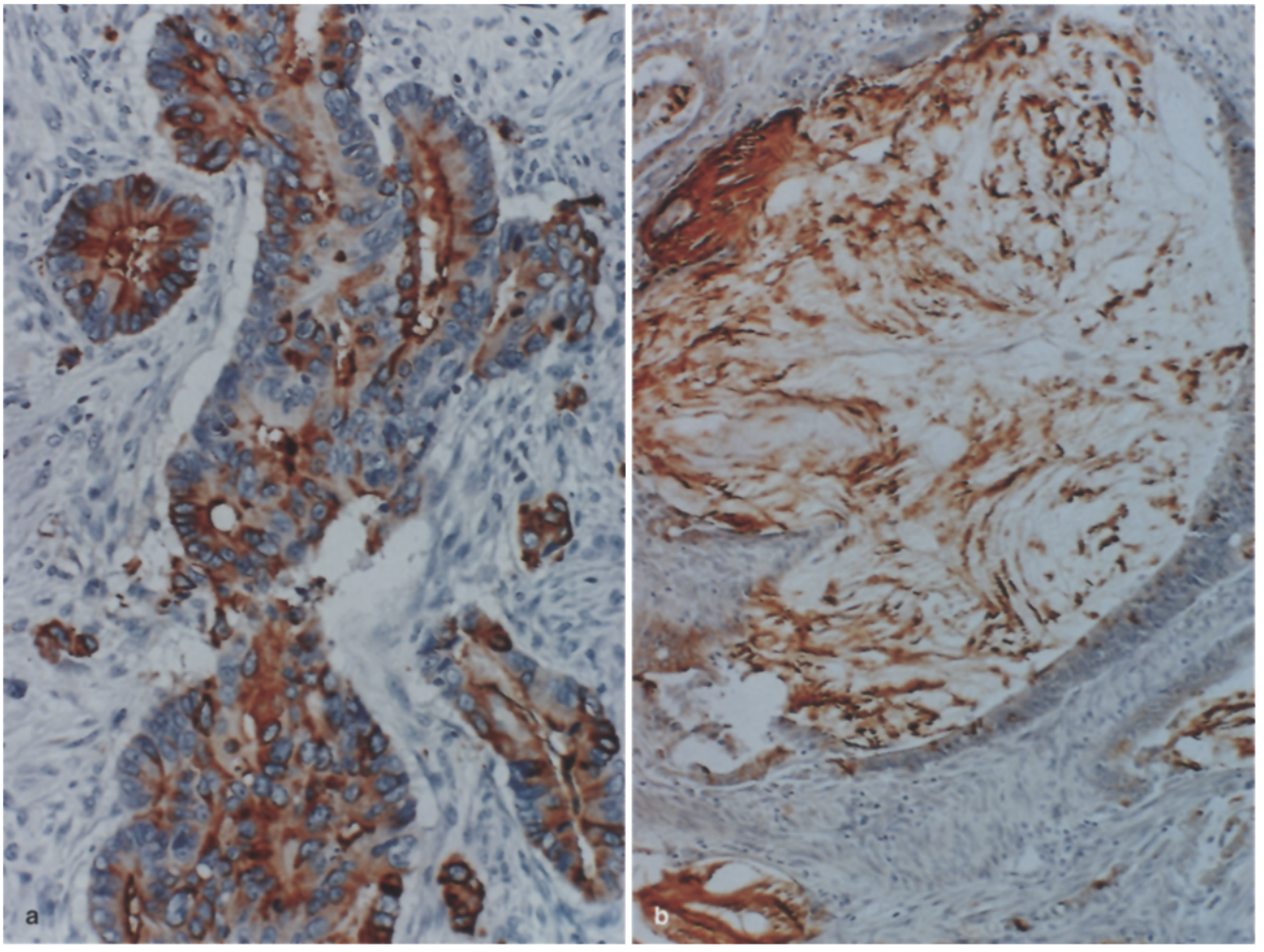


Fig. 1a,b Monoclonal antibody anti-Tn antigen. **a** In neoplastic elements of hereditary nonpolyposis colorectal cancer (HNPCC) the reactivity is present in the cytoplasm, at the cell membrane and in the glandular secretion. ×50. **b** Strong positivity of glandular secretion in a mucinous sporadic CRC. ×25

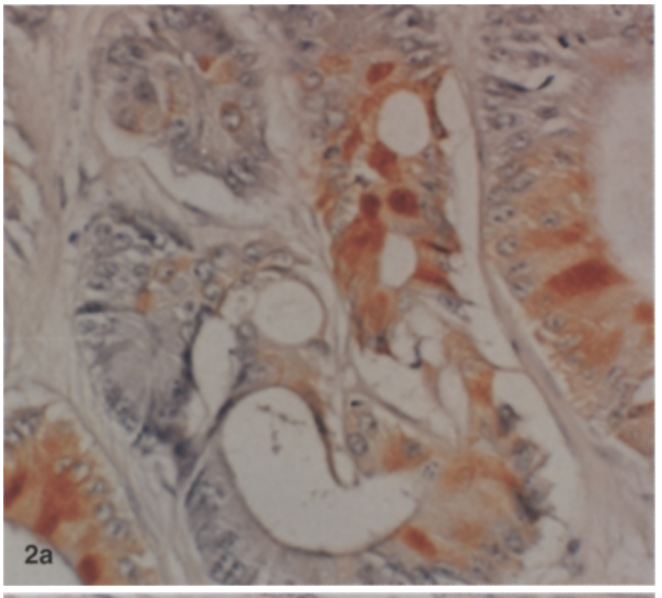
ly a statistically significant difference with $\chi^2 = 5.243$ and $P = 0.022$). In reactive cases, moderate to strong staining intensity was localized at the luminal cell membrane (HNPCC: 77.8%; sporadic CRC: 100%), in glandular secretions (HNPCC: 100%; sporadic CRC: 80%) and occasionally in the cytoplasm (Fig. 3a). In addition, vacuoles of neoplastic elements that exhibited goblet cell differentiation showed a variable degree of reactivity (Fig. 3b), which was better evident in HNPCC (33.3% of cases). Mucinous carcinomas in HNPCC only exhibited strong positivity for intraglandular mucus in 3 of 6 cases (Fig. 3c).

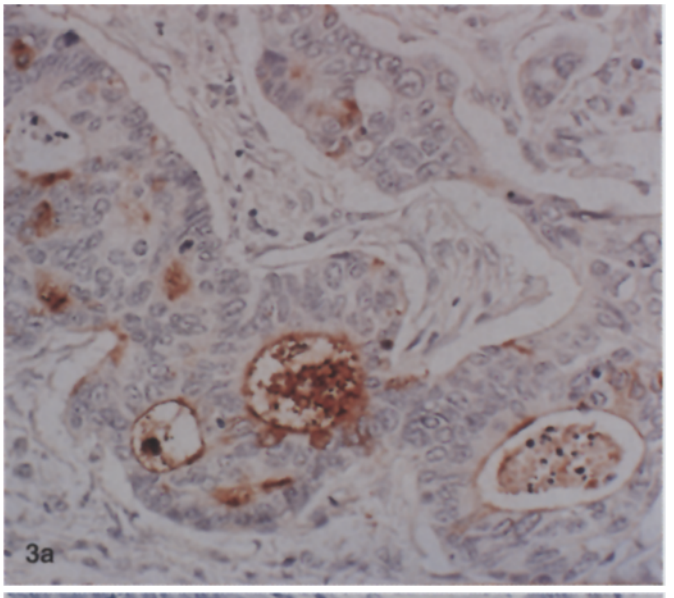
Normal colonic mucosa specimens were always positive for ACA, with a different pattern of expression depending on the segment of the large bowel examined. In right-sided colonic mucosa weak to moderate staining intensity was encountered in the luminal cell membrane (glycocalyx region) and in the apical cytoplasmic region along the entire crypts; moreover, in 66.7% of cases moderate reactivity was evident in vacuoles of goblet cells present in the upper half of the crypts and at the surface epithelium. In the left portion of colon and in the rectum moderate positivity was found in the luminal membrane and in the apical cytoplasmic region of the cells localized in the lower part of the crypts, while in 1 case a pattern similar to that exhibited by right colonic samples was encountered. In addition, a weak to moderate staining intensity of the glandular secretion was present in 60% of right- and 53.3% of left-sided large bowel specimens examined.

In HNPCC and sporadic CRC reactivity was present in 100% and 90% of cases, respectively. In reactive

Fig. 2a–c Monoclonal antibody anti-STn antigen. **a** Reactive neoplastic cells intermingled with negative ones in a case of HNPCC. ×50. **b** In a sporadic colorectal cancer (CRC) the reactivity is mainly localized at the luminal cell membrane and in glandular secretion. ×100. **c** A mucinous HNPCC shows strong positivity of intraglandular mucus. ×50 ▶

Fig. 3a–c Monoclonal antibody anti-T antigen. **a** The reactivity is localized at the luminal cell membrane, in glandular secretion and occasionally in the cytoplasm in a sporadic CRC. ×50. **b** Reactivity is evident in vacuoles of HNPCC elements that exhibit goblet cell differentiation. ×50. **c** Strong positivity of intraglandular mucus of a mucinous HNPCC. ×50


2a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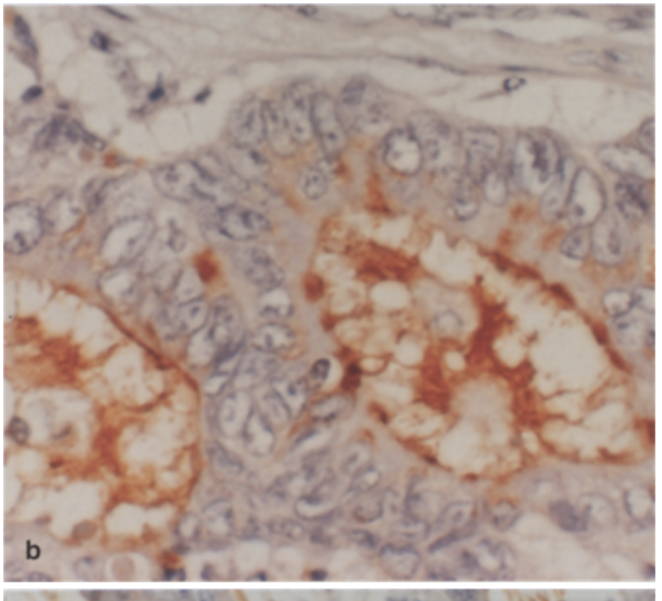
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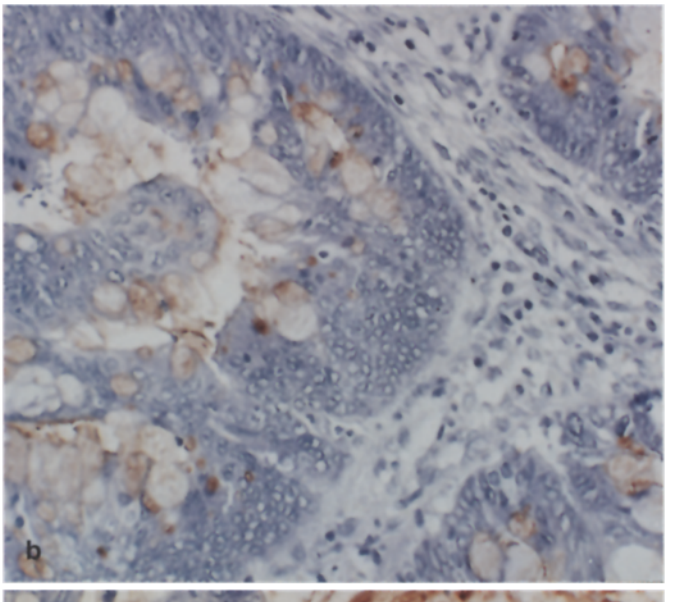
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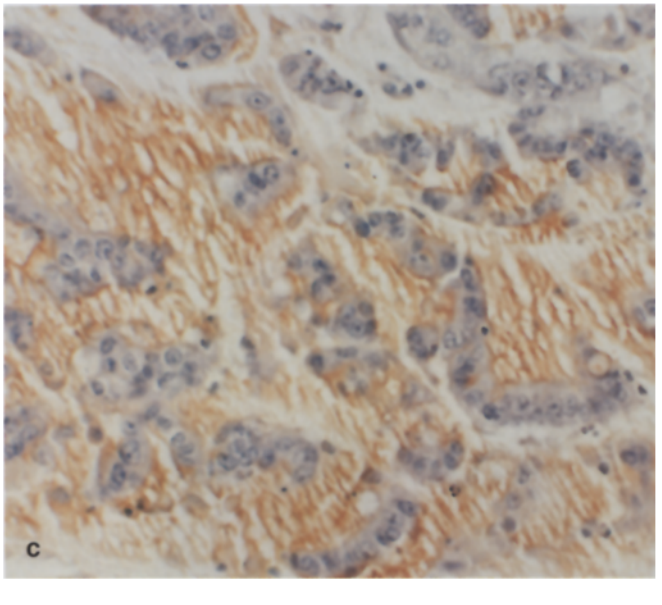
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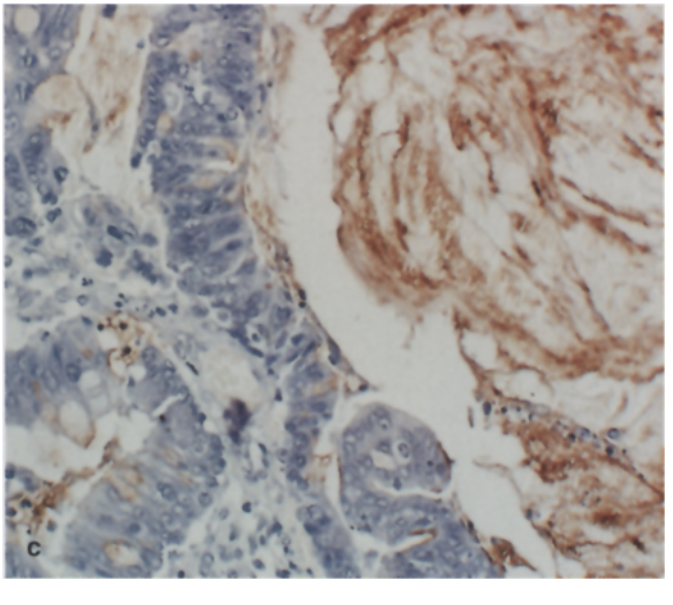
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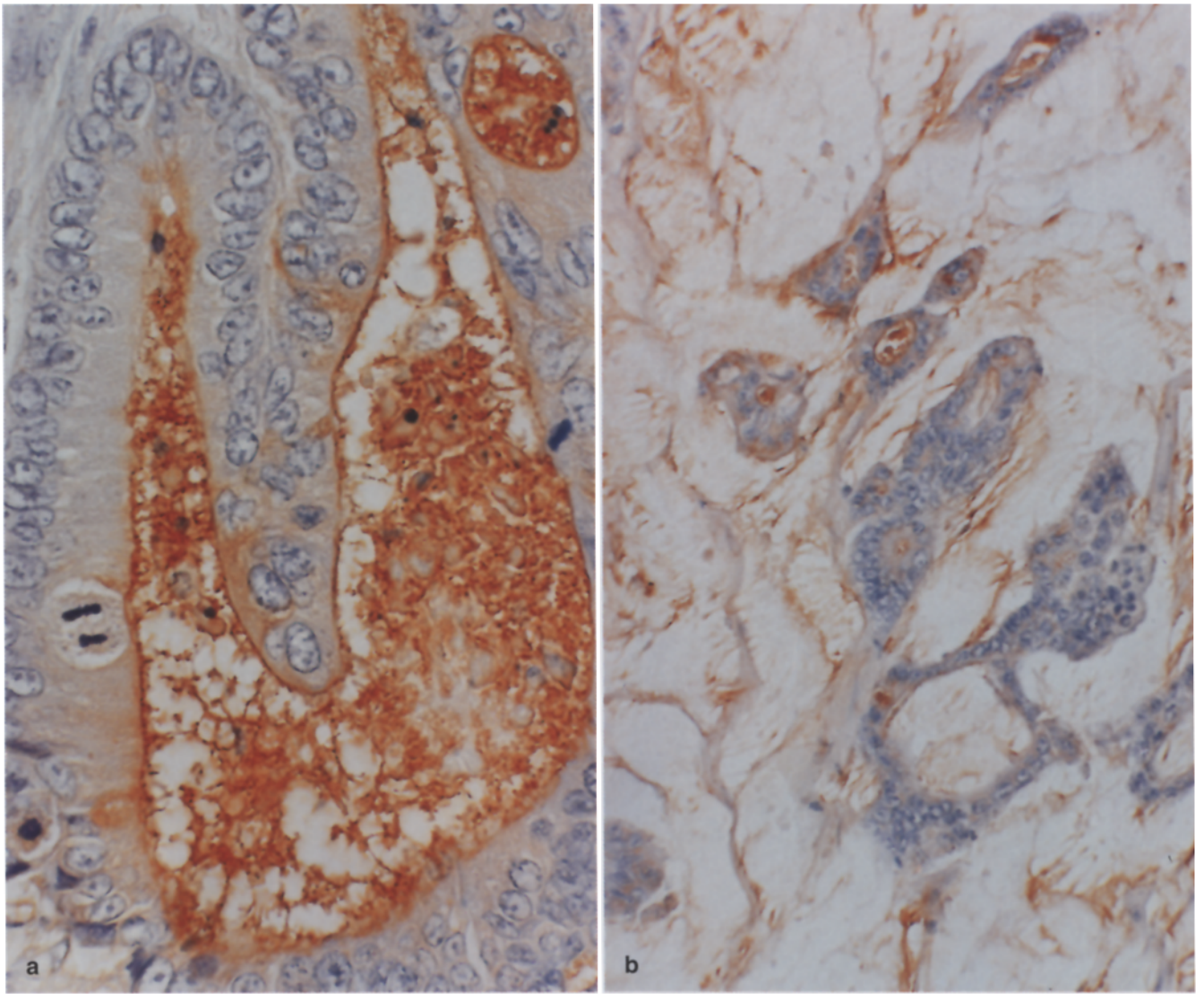

Fig. 4a,b *Amaranthus caudatus* agglutinin. **a** Strong positivity of luminal cell membrane and glandular secretion together with a moderate cytoplasmic reactivity in a sporadic CRC. ×100. **b** A mucinous HNPCC shows positivity of cells and of the intraglandular mucus. ×50

cases, a weak to strong staining intensity was localized in the cytoplasm (HNPCC: 93.3%; sporadic CRC: 55.5%), at the luminal cell membrane (HNPCC: 100%; sporadic CRC: 94.4%) and in glandular secretions (HNPCC: 86.7%; sporadic CRC: 61.1) (Fig. 4a, b). The reactivity was homogeneous in HNPCC cases, while an evident heterogeneity in staining score was exhibited in sporadic CRC (Table 3).

The percentage and number of HNPCC and sporadic CRC cases grouped for the simultaneous reactivity of HB-Tn1, HB-STn1, HB-T1 and ACA are reported in Table 4.

Table 4 Simultaneous reactivity for HB-Tn1, HB-Stn1, HB-T1 and ACA in HNPCC and in sporadic CRC

	HB-Tn1	HB-STn1	HB-T1	ACA	Cases (%)
HNPCC	+	+	+	+	40% (6/15)
	+	+	−	+	33.3% (5/15)
	+	+	−	−	0
	+	−	−	+	6.7% (1/15)
	+	−	+	+	20% (3/15)
Sporadic CRC	+	+	+	+	25% (15/60)
	+	+	−	+	60% (36/60)
	+	+	−	−	10% (6/60)
	+	−	−	+	5% (3/60)
	+	−	+	+	0

Discussion

In the present study we have demonstrated different expression of four simple mucin-type carbohydrate antigens in normal colonic mucosa, sporadic CRC and HNPCC.

In normal colonic mucosa constant reactivity was encountered only for Tn and the cryptic sialylated form of T, while negative results were always obtained for sialosyl-Tn and T antigens. The immunoexpression of Tn was mainly localized within the Golgi zone of enterocytes, in which glycosylation of apomucin is initiated, while the unreactivity of goblet cell mucins is due to a masking by further glycosylation of this antigen. Our findings are similar to those reported in the literature [17, 37], suggesting that Tn cannot be regarded as a specific marker for CRC, as previously suggested [11]. Moreover, in our cases of normal colonic mucosa, the T antigen was present only in its cryptic sialylated form, as documented by the constant reactivity for ACA associated with HB-T1 negativity. Furthermore, with ACA we have demonstrated, as have others [32], that there is a regional difference in staining pattern between the right and the left colon, which is probably related to their different physiological functions. With regard to the sialosyl-Tn antigen, the nonreactivity encountered in our cases of normal colonic mucosa is in agreement to that reported elsewhere[11, 12, 14] and has been related to the heavy *O*-acetylation of sialic acid with the formation of a pseudocryptic form of sialosyl-Tn antigen [16, 17]. On the other hand, in normal colonic mucosa, a variable presence of sialosyl-Tn has been demonstrated by others [19, 24, 37], as a result of the production of non-*O*-acetylated sialic acid [16, 17].

In our cases of sporadic CRC or HNPCC, a variable pattern of positivity for Tn, sialosyl-Tn, T and 'cryptic' T antigen has been documented. In particular, the Tn antigen, always ubiquitously distributed in the cytoplasm and sometimes on luminal cell membranes and/or in glandular secretion, may suggest overproduction of this antigen, perhaps determined by incomplete glycosylation. Nevertheless, the coexpression of Tn along with sialosyl-Tn, T and cryptic T antigens may indicate that biosynthetic pathways necessary for further glycosylation of Tn are active in CRC. Nevertheless, an upregulation of membrane-associated MUC1 glycoproteins cannot be excluded to explain the Tn expression in CRC [17]. The cryptic form of T antigen expression has already been investigated by others [1, 32]; we have documented more diffuse reactivity in the cytoplasm of neoplastic cells than in normal elements, probably due to an altered cytoplasmatic transit of this antigen during its biosynthetic pathway. Another interesting finding is the immunocytochemical positivity of sialosyl-Tn and T antigens, which is only evident in CRC. In particular, sialosyl-Tn antigen has been described as a highly sensitive and specific marker for CRC [11, 12, 14], and its reactivity may be explained by a de novo expression of an α2,6 sialyltransferase [31] and/or by the loss of sialic acid *O*-acetyl substituents [16, 17]. A rapid intracellular turnover of sialosyl-Tn antigen may also be assumed, since positivity of intraglandular mucus was observed in some cases of mucinous carcinomas. Moreover, our data on T antigen expression in neoplastic cells indicates the activity of β1,3 galactosyltransferase, an enzyme that is involved in a glycosylation pathway not verified in normal colonocytes [26, 27]. However, regarding the T antigen expression in CRC, the use of antibodies and lectins with different specificity is probably the main reason for the conflicting results reported in the literature [2, 11, 17, 18, 20, 23, 24, 32, 43].

The most important finding of the present study is the quantitative difference encountered in the expression of sialosyl-Tn and T antigens when sporadic CRC and HNPCC cases were compared. In neoplastic elements, the sialosyl-Tn antigen has been demonstrated in a higher percentage ($P = 0.037$) of sporadic CRC than of HNPCC cases, suggesting a relevant α2,6 sialyltransferase activity in sporadic CRC. However, the expression of T antigen was significantly higher ($P = 0.022$) in HNPCC, indicating an evident β1,3 galactosyltransferase activity, compared with sporadic CRC cases. Nevertheless, when the simultaneous expression of HB-Tn1, HB-STn1, HB-T1 and ACA was analysed case by case, we found the coexpression of HB-T1 and ACA in 26.7% of HNPCC and in 5% of sporadic CRC cases, suggesting exclusive galactosylation of Tn antigen with the inactivity of α2,6 sialyltransferase. In contrast, the above-mentioned enzyme activity has not been utilized as an exclusive pathway in HNPCC, while 10% of sporadic CRC cases showed the α2,6 sialyltransferase pathway exclusively.

None of the antigens investigated allows us to identify a peculiar phenotype useful to distinguish HNPCC from sporadic CRC; more extensive investigations are needed to confirm the preferential expression of T antigen in HNPCC and to explain its biological significance.

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