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Stromelysin-3 expression in early (pT1) carcinomas and pseudoinvasive lesions of the colorectum

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Abstract Pseudoinvasion in colorectal adenomas is often difficult to distinguish from invasive carcinoma. Previous studies have indicated that expression of stromelysin-3 (ST-3), one of the metalloproteinase family of enzymes, may be useful for the identification of early invasive carcinoma. The goal of our study was to detect ST-3 expression in colorectal adenomatous polyps to see if it could be helpful for the differential diagnosis of pseudoinvasion vs. true invasion. We studied 25 polypectomy specimens which were divided histologically into 2 groups; the first consisted of 15 adenomas with invasive carcinoma, 8 of these carcinomas were more diffusely infiltrative (pT1), and 7 tended to be expansively invasive. The second group was composed of 10 adenomas with pseudoinvasion. A ^{35}S labelled cDNA probe was used for in situ hybridization (ISH) and a monoclonal antibody (5ST-4A9) for immunohistochemistry (IHC). The distribution of ST-3 expression as detected by IHC and ISH was identical. All diffusely infiltrative carcinoma cases showed ST-3 expression, but only focally in 2 cases with marked lymphocytic infiltration. None of the expansive carcinoma or pseudoinvasion cases showed ST-3 expression. ST-3 expression seems to be an indicator of invasion, but a negative reaction for ST-3 does not rule out an expansive invasive neoplasm or a diffusely infiltrative invasive tumour with a dense lymphocytic reaction.

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Introduction

Pseudoinvasion in colonic adenomatous polyps is a well-recognized phenomenon in which benign glands are found within the colonic submucosa simulating invasion by an adenocarcinoma. Pseudoinvasion is estimated to occur in as many as 2–10% [10, 16, 19] of all adenomatous polyps. With the increased use of endoscopic procedures for the removal of colonic polyps, pathologists are increasingly confronted with the differential diagnosis of pseudoinvasion vs true invasion in these lesions. Misdiagnosis of pseudoinvasion as true invasion can lead to unnecessary surgery while, conversely, truly invasive lesions misdiagnosed as pseudoinvasion may result in a missed opportunity to treat a colon carcinoma successfully with a limited surgical procedure.

Owing to the importance of this distinction and its difficulty in many cases, a variety of histological criteria have been developed to aid in this differential diagnosis. Features [3, 16, 19] that favour the diagnosis of pseudoinvasion include lack of stromal desmoplasia, haemosiderin deposition in the vicinity and lack of high-grade dysplasia in the submucosal gland clusters. While high-grade dysplasia is said to be the most reliable indicator for the diagnosis of true invasion, this has been complicated by a lack of consensus on the definition of high-grade dysplasia [4] and the description of cases of highly dysplastic glands in pseudoinvasion [17].

Stromelysin-3 (ST-3), first described in 1990 [1], is a member of the metalloproteinase family of proteases, which also includes collagenases and gelatinases. Metalloproteinases have been found to have important roles in the degradation of the extracellular matrix (ECM) during the process of tumour invasion and also in wound healing and embryonic development. In contrast to other members of the metalloproteinase family, ST-3 expres-

Table 1 Clinicopathologic characteristics and results of immunohistochemistry (*IHC*) and in situ hybridization (ISH) for stromelysin-3 (*ST*-3) in invasive pT1 colon carcinomas (*TA* tubular adenoma, *TVA* tubulovillous adenoma, *T* T category (according to the UICC), *G*- degree of differentiation, *N* metastases to regional lymph nodes (according to the UICC, +++ strongly positive, + weakly positive, 0 negative, *x* unknown)

Case no.	Sex	Age	Specimen	Site	Size (cm)	Type	Pattern	T	G	N	IHC	ISH
1	F	71	Polypectomy	Sigmoid	1.5	TA	Diffuse	1	2	x	+++	+++
2	M	59	Polypectomy	Sigmoid	2.0	TA	Diffuse	1	1	x	+	+
3	M	55	Resection	Ascending	1.3	TA	Diffuse	1	2	1	+++	+++
4	M	75	Polypectomy	Sigmoid	2.6	TA	Diffuse	1	2	x	+++	+++
5	M	67	Polypectomy	Sigmoid	3.0	TA	Diffuse	1	2	x	+	+
6	F	61	Polypectomy	Rectum	1.3	TVA	Diffuse	1	2	x	+++	+++
7	M	70	Biopsy	Sigmoid	4.5	TVA	Diffuse	1	2	x	+++	+++
8	F	68	Polypectomy	Sigmoid	1.0	TA	Diffuse	1	1	x	+++	+++
9	M	55	Polypectomy	Sigmoid	1.5	TVA	Expansive	1	2	x	0	0
10	M	73	Polypectomy	Sigmoid	1.2	TA	Expansive	1	1	x	0	0
11	M	42	Polypectomy	Sigmoid	2.0	TA	Expansive	1	1	x	0	0
12	F	75	Polypectomy	Sigmoid	1.3	TA	Expansive	1	1	x	0	0
13	M	49	Polypectomy	Sigmoid	2.2	TVA	Expansive	1	2	x	0	0
14	F	59	Polypectomy	Sigmoid	1.3	TA	Expansive	1	1	x	0	0
15	M	80	Polypectomy	Sigmoid	1.3	TVA	Expansive	1	1	x	0	0

sion has not been found in tumour cells but is seen only within stromal fibroblasts in the immediate vicinity of invasive tumours. ST-3 has been detected in carcinomas of the breast [11, 27], lung [24], colon [22, 25] and head and neck [18], and in basal cell carcinomas (BCC) of the skin [26]. In head and neck carcinoma and BCC, ST-3 was found to be especially strongly expressed in those tumours that had a pronounced locally invasive growth pattern [15]. ST-3 is particularly interesting as a potential diagnostic marker for invasion, since its expression has been described in preinvasive and early invasive carcinomas [2] in addition to frankly invasive tumours.

The goal of our study was to localize ST-3 using immunohistochemistry and in situ hybridization, to see whether its expression might prove to be a helpful addition to the present histological criteria for the distinction between pseudoinvasion and true invasion in colorectal adenomas.

Materials and methods

The specimens for study included 25 polypectomy specimens from 24 patients of the Institute of Pathology of Bayreuth Hospital (Table 1), 18 of whom were male and 6, female. Almost all, 23, of the specimens came from the sigmoid colon, 1 from the ascending colon and 1 from the rectum. The specimens were formalin fixed, embedded in paraffin and cut in 6-μm-thick sections. Diagnoses of invasion or pseudoinvasion were made according to established criteria [16, 19] and the carcinomas were staged according to the criteria of the UICC [23] on haematoxylin and eosin-stained slides.

On the basis of histology, the specimens were divided into two groups as follows: 15 specimens were stalk invasive (pT1), and 10 specimens were diagnosed as adenomatous polyps with pseudoinvasion (Fig. 3). Among the invasive carcinomas, 8 cases tended to have a diffusely infiltrative pattern (Fig. 1), and 7 a predominantly expansive growth pattern (Fig. 2). As in a previous study of gastric carcinoma [7], an expansive growth pattern was defined as a smooth tumour–stroma border at the invasive front without tumour cell disassociation, while a diffusely infiltrative pattern was defined as an irregular tumour–stroma border with tumour cell dissociation. For the purposes of this study, only cases with clear-cut histological diagnoses and separation into these two carcinoma growth patterns were used. Serial sections in our expansive-type carcinoma cases failed to demonstrate a connection to the overlying surface which could have justified a classification of high-grade dysplasia in pseudoinvasion.

A 118-bp-long ^{35}S cDNA-labelled probe for ST-3 ISH was produced by reverse transcription of a cRNA template that had been transcribed from plasmid DNA (kindly provided by Prof. P. Chambon, Strasbourg) using a T3 (sense) or T7 (antisense) RNA polymerase (Stratagene, Heidelberg, Germany). In situ hybridization was then performed on paraffin sections using a modification of the method of Hogan [14].

The paraffin sections were cut onto 2% silanized slides and then rehydrated in PBS and digested in proteinase K (1 μg/ml proteinase K in 100 mM Tris (pH 8.0), 50 mM EDTA) for 13 min at 37°C. Proteinase K was inactivated by fixation with 4% paraformaldehyde in PBS for 20 min at room temperature. Sections were acetylated for 10 min at room temperature in 0.25% acetic anhydride and 100 mM triethanolamine (pH 8.0). Hybridization was carried out using the 35S labelled cDNA probe in a buffer containing 50% deionized formamide, 300 mM NaCl, 10 mM Tris HCl, 10 mM $NaP0_4$ (pH 6.8), 5 mM EDTA, 10% dextran sulfate, 1 μg/ml of tRNA, 10 mM dithiothreitol (DTT), Ficoll 400, 0.02% polyvinylpyrolidone, and 0.02% BSA. Sections were hybridized at 52°C for 12–16 h in a humidified chamber. High-stringency washing (3×2 h at 52°C in the presence of 300 mM NaCl, 10 mM Tris HCl, 10 mM $NaP0_4$ (pH 6.8), 5 mM EDTA, 50% deionized formamide, 10 mM DTT) was followed by dehydration in ethanol containing 300 mM ammonium acetate. For autoradiography, slides were coated with Kodak NTB2 film emulsion and exposed for 10 days. After development, the sections were counterstained with haematoxylin.

Immunohistochemistry was performed using a monoclonal antibody (5ST-4A9 [20], also kindly provided by Prof. P. Chambon, Strasbourg) at a 1:2000 dilution followed by development with either Avidin–Biotin or APAAP as previously described. Negative controls consisted of omission of the primary antibody and were consistently negative. A case known to be positive for ST-3 was included with each set of immunohistochemical and in situ hybridization reactions as a positive control.

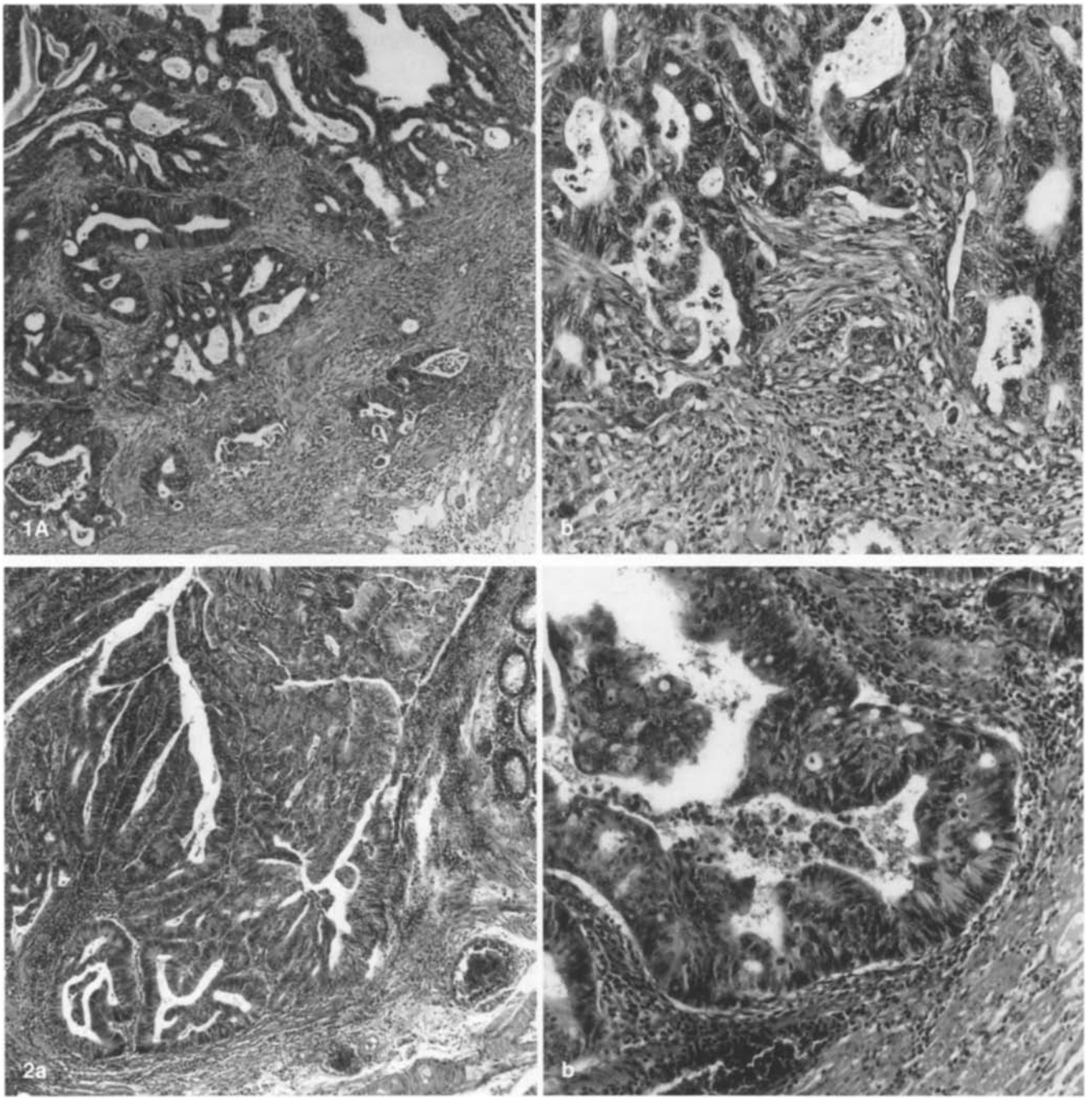

Fig. 1a, b Invasive carcinoma with an diffusely infiltrative pattern in a colon adenoma. The high-power magnification (**b**) shows an irregular tumour–stroma border with nests and individual tumour cells infiltrating the stroma. HE, original magnification **a** ×50.4, **b** ×320

Fig. 2a, b Invasive carcinoma with an expansive pattern in a colon adenoma. In contrast to the diffusely infiltrative pattern, the tumour–stroma interface (**b**) shows a smooth pushing border. HE, original magnification **a** ×50.4, **b** ×320

Results

The results of in situ hybridization (ISH) and immunohistochemistry (ICH) for ST-3 were identical in all cases (Tables 1, 2). The 8 pT1 carcinomas which tended to have a diffusely infiltrative growth pattern included 6 that were strongly positive for ST-3 (Figs. 4, 5a) in the fibroblasts immediately adjacent to nests of invasive tumour in the submucosa. The remaining 2 cases, both of which had a marked lymphocytic infiltrate at the invasion front, were only focally positive.

All the 7 invasive carcinoma cases that tended to have an expansive growth pattern (Fig. 5b) were negative for ST-3 by both IHC and ISH. The 10 cases of pseudoinva-

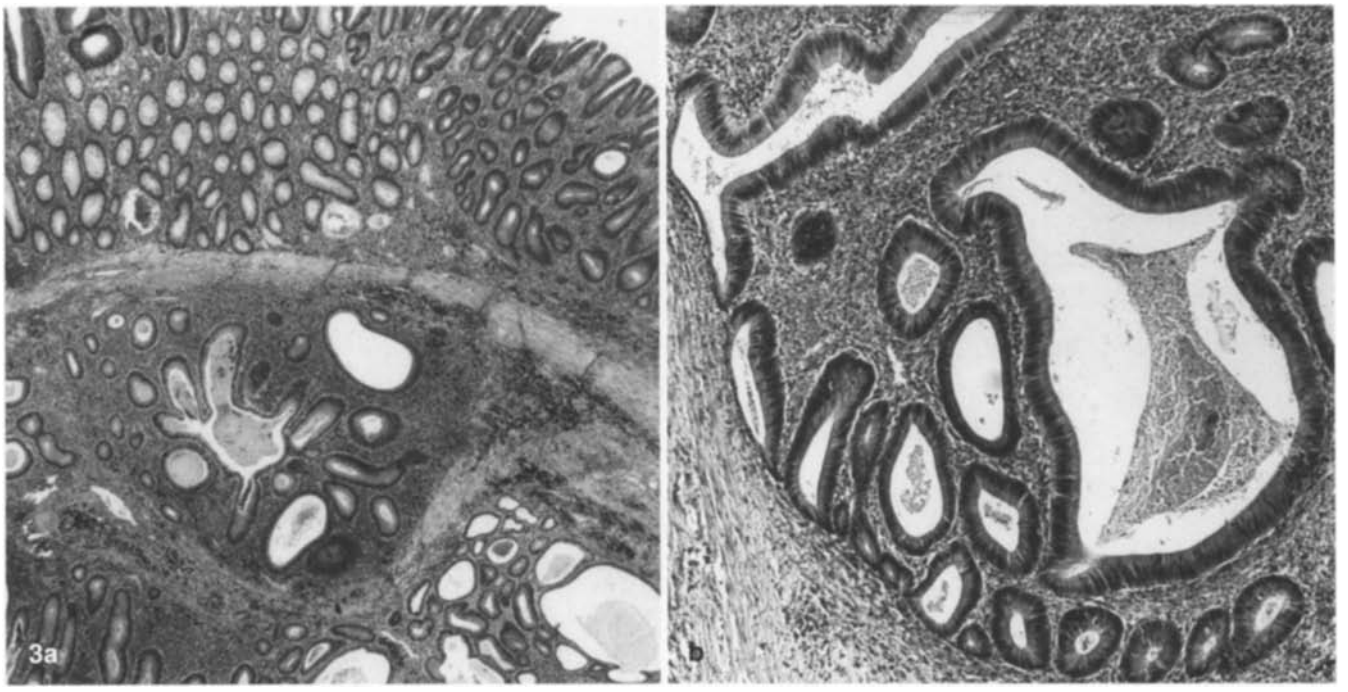

Fig. 3a, b An adenoma with submucosal pseudoinvasive glands. Several of the classic histological features of pseudoinvasion, including cystic gland dilatation, haemosiderin deposition and areas of periglandular lamina propria are present. HE, original magnification **a** ×25.6, **b** ×200

sion in adenomatous polyps were also all negative for ST-3.

Discussion

Pseudoinvasion in adenomatous colorectal polyps is a well-recognized phenomenon, which over the years has also been called colitis cystica profunda ex adenoma [6], epithelial misplacement [10], pseudocarcinoma and pseudocarcinomatous invasion [5]. It is relatively common, especially compared with the number of adenomatous polyps that show true invasion. In one study [16], of a series of 2341 adenomatous polyps, 110 had areas of true invasion, as opposed to 54 adenomatous polyps with pseudoinvasion. The number of cases and therefore the frequency with which the pathologist is confronted with the differential diagnosis of pseudoinvasion vs true invasion can be expected to rise in the future with the increasing use of endoscopic polypectomy.

Pseudoinvasion is thought to result from the herniation of non-malignant epithelium through the muscularis mucosa [17], which has been shown to be discontinuous in the colon [19]. Such islands of mucosa within the submucosa may lose their connection to the surface ("true epithelial misplacement") or maintain a connection ("false epithelial misplacement") that can often only be distinguished by multiple serial sections.

The histological criteria for the diagnosis of pseudoinvasion cause difficulty because of the possibility that carcinomas may also have several of the features described, including cystically dilated glands with mucin retention, stromal haemorrhage with haemosiderin deposition and, as in our group of carcinomas with an expansive growth pattern, a non-diffusely infiltrative invasion front. Further, while the lack of high-grade dysplasia, or at least of a higher degree of dysplasia than the overlying polyp surface, is considered by many authors to be a

Table 2 Clinicopathological characteristics and results of IHC and ISH for ST-3 in polyps with pseudoinvasion

Case	Sex	Age	Specimen	Site	Size	Type	Dysplasia	IHC	ISH
1	M	73	Polypectomy	Sigmoid	1.2	TA	Low	0	0
2	M	65	Polypectomy	Sigmoid	1.5	TA	Low	0	0
3	M	77	Polypectomy	Sigmoid	1.3	TVA	High	0	0
4	M	66	Polypectomy	Sigmoid	0.9	TA	Low	0	0
5	M	65	Polypectomy	Sigmoid	1.4	TA	Moderate	0	0
6	M	56	Polypectomy	Sigmoid	1.6	TA	Low	0	0
7	M	59	Polypectomy	Sigmoid	1.2	TA	Moderate	0	0
8	M	66	Polypectomy	Sigmoid	2.0	TVA	Low	0	0
9	F	70	Polypectomy	Sigmoid	1.4	TA	Low	0	0
10	M	51	Polypectomy	Sigmoid	1.4	TA	Low	0	0

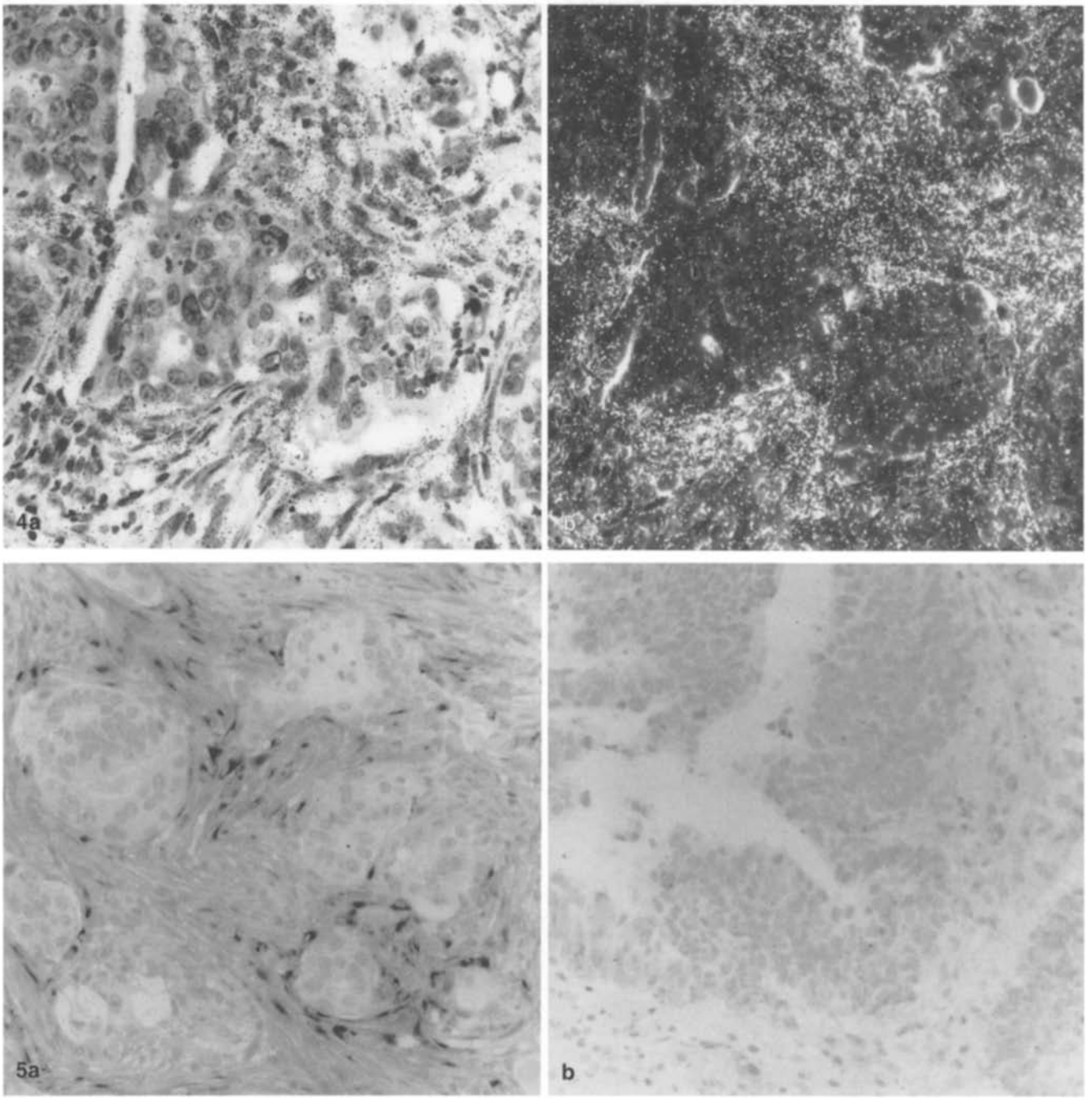

Fig. 4a, b A diffusely infiltrative carcinoma, positive for ST-3 by in situ hybridization (**a** bright field, **b** dark field). The fibroblasts directly adjacent to the infiltrating tumour nests show a strong hybridization signal. Original magnification ×320

Fig. 5a, b Immunohistochemistry for ST-3 (APAAP development method). **a** A diffusely infiltrative carcinoma. The surrounding stromal fibroblasts show a strongly positive cytoplasmic reaction for ST-3, with the same distribution as seen by in situ hybridization. **b** An expansive-type carcinoma, negative for ST-3 using the same method. Original magnification ×160

very valuable criterion of pseudoinvasion [10], cases of pseudoinvasion with high-grade dysplasia have been described in the literature [17]. The most reliable criterion of pseudoinvasion in questionable cases is said to be the demonstration of a connection to the surface; this may require the examination of numerous serial sections.

Previous studies [27] had led us to expect that ST-3 expression might have a useful role in the differential diagnosis of true vs pseudoinvasion. It is topographically restricted in its expression to fibroblasts directly adjacent to malignant tumours (unlike other metalloproteinases and serine proteases, which show a much more diffuse pattern of expression), and this allows for a more precise identification of invasive areas. While all cases of pseu-

doinvasion were negative and all cases of carcinoma with a more diffusely infiltrative growth pattern expressed ST-3, all the cases with a predominantly expansive pattern that had been diagnosed as carcinoma by established criteria were negative for ST-3 by both IHC and ISH. Therefore, while a positive reaction seems to be an indication of invasion, lack of ST-3 detection does not rule out invasion. ST-3 expression is thus not sufficiently sensitive to be a useful diagnostic marker in the diagnosis of early invasive carcinoma of the colon.

However, ST-3 appears to be a potentially useful marker for the study of the relationship between tumour invasion biology in its early stages and histopathology. As one might expect from their histology, a majority of the diffusely infiltrative tumours had a level of expression of ST-3 that correlates with a high degree of proteolytic enzyme activity and degradation of the ECM. In contrast, the lack of expression in carcinomas with a more expansive growth pattern is a sign that they have a mechanism of invasion that can be said to be more "passive", with a less important role for the degradation of the ECM by proteolytic enzymes. It would also be interesting to see whether these two invasive growth patterns might have differing patterns of cell adhesion molecule expression, since previous studies of integrins [13] and E-cadherin [8] have shown a tendency towards stronger expression of these molecules in areas of colonic tumours with more highly organized gland formation.

It is conceivable that expansive-type carcinoma is present in the submucosa as a result of a process of herniation similar to the mechanism thought to be responsible for pseudoinvasion. Some authors maintain that if such a connection to the surface could be demonstrated, these lesions might be considered to be "high-grade dysplasia in pseudoinvasion" [17]. The possibility cannot be completely excluded that some of our expansive-type carcinomas had a connection to the overlying mucosa, which we were not able to demonstrate. From a practical point of view it is probably preferable to classify such lesions as invasive cancer since, even if they are not invasive cancers, these submucosal high-grade dysplastic glands have an uncertain biological potential. It is unknown whether such lesions might progress to carcinoma, or might contribute to local recurrence of removed adenomatous polyps, obstruction or, potentially, may even spread into the peritoneal cavity and form pseudomyxoma. According to our results, the pattern of ST-3 expression does not appear to be able to distinguish between these entities and it indicates that their relation to the surrounding stroma may be biologically similar.

An unexpected result was the very weak expression of ST-3 in diffusely infiltrative carcinomas in areas with a dense lymphocytic infiltrate. It is difficult to explain this finding, except to speculate that the interaction between tumour cells and their stroma may be modulated by the reaction of the immune system. Whether this has any connection with the observation that colon carcinomas with dense [21] or particular patterns of lymphocytic infiltration (so-called Crohn's-like infiltrate [9, 12] have been shown to indicate a better prognosis will only be disclosed by further study of ST-3 expression in such types of tumours compared with survival data.

In conclusion, although ST-3 was negative in all cases of pseudoinvasion, its negativity in the group of invasive carcinomas with a more expansive growth pattern appears to preclude its use as a sensitive indicator of invasion. However, the presence or absence of ST-3 in the invasive cancers seems to be a reflection of the tumour's capacity for active degradation of the ECM and therefore of its local aggressiveness. Further studies with a larger number of cases and detailed clinical follow-up are necessary to show whether ST-3 is a useful marker for the prognostic assessment of these early invasive colorectal cancers.

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