Virchows Arch (1999) 434:489–495

ORIGINAL ARTICLE

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Loss of immunohistochemical E-cadherin expression in colon cancer is not due to structural gene alterations

Received: 14 September 1998 / Accepted: 23 February 1999

Abstract E-cadherin, a transmembrane cell adhesion molecule, has been observed to have an altered pattern of immunoreactivity in several types of carcinomas. In lobular breast cancer, loss of immunoreactivity has been shown to be due either to out-of-frame deletions or to nonsense mutations of the E-cadherin gene. We analysed 29 cases of completely resected colon carcinoma with immunohistochemistry using the HEC-D1 antibody. Normal protein expression similar to that in the adjacent nonmalignant mucosa was seen in 6 cases, whereas 23 tumours had reduced or absent E-cadherin expression. In the 8 cases with no expression of E-cadherin revealed by immunohistochemistry, the entire E-cadherin cDNA sequence was analysed. In these cases, sequence analysis failed to reveal any cDNA mutations despite the negative immunohistochemistry. Possible explanations for this discrepancy include regulatory defects in the E-cadherin promoter, abnormalities at the translation or protein processing levels and mutations in other parts of the gene that were not investigated by the cDNA analysis (e.g. intronic sequences), which could play a role in causing abnormal processing of the E-cadherin protein.

Key words E-cadherin · Colorectal cancer · Immunohistochemistry · Mutation

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Introduction

Cell-cell interactions are of major importance in tissue development and for the maintenance of normal organ function. Malignant transformation of tissue is characterized by cellular disintegration and dissemination leading to invasive growth. E-cadherin, a calcium-ion dependent, cell-cell adhesion molecule, has a key role in the organization of epithelia, the morphological appearance of epithelial cells and their ability to form stable aggregates. Transfection of various cadherins into mesenchymal cells results in an epithelioid morphology in which the previously separate cells form aggregates. Homotypic cellular aggregates of various cell types have been shown to be mediated by different classes of cadherins, demonstrating that the members of the cadherin family have a selective ability to form cell-cell interactions [6, 28, 32, 33].

Histopathological examination of human carcinomas has revealed inconsistencies in the correlation of tumour stage and grade with reduced immunoreactivity for E-cadherin [4, 15, 19, 20, 24, 27, 35]. Studies of colorectal neoplasms have suggested that there is a negative correlation between the degree of E-cadherin expression and size and severe dysplasia in adenomas and between E-cadherin expression and tumour grade and the occurrence of metastasis in carcinomas [7, 9]. In several other studies, however, the pattern of E-cadherin expression showed no correlation with conventional staging (Dukes' classification) [8] or tumour differentiation [17, 23, 34]. Loss of E-cadherin expression has been correlated with an increased rate of lymph node metastases in some studies [27, 29], presumably because of increased cell motility resulting from the release of the cells from their attachment to adjacent epithelial cells.

A possible explanation for reduced expression and loss of biological function of E-cadherin has come from the analysis of the E-cadherin gene. In gastric carcinoma loss of E-cadherin expression has been shown to be correlated with a lack of differentiation and with the diffuse histopathological subtype according to the Laurén classification. The diffuse type, which is characterized by inf-

iltrative growth, has been shown to have lower E-cadherin immunoreactivity than nontumour mucosa [21, 36]. Genomic E-cadherin alterations (point mutations or splice-site mutations at the DNA level, with resulting exon loss) have been reported in 50% of diffuse-type gastric carcinomas [1]. Exon deletions have also been detected in a Japanese patient population [22]. In contrast, lobular breast carcinoma, which also has a diffuse infiltrative pattern, has mutations of the E-cadherin gene, but these are nonsense mutations, which result in interruption of protein synthesis [2].

Since the previously published studies describing abnormalities of E-cadherin in colorectal carcinoma are mainly based on immunohistochemistry and do not describe their underlying genomic basis, our aim was to determine whether they could be traced to mutations of the E-cadherin gene.

Materials and methods

Samples of carcinoma and normal mucosa from 29 patients who had undergone surgery for adenocarcinoma of the colon were obtained and snap frozen in liquid nitrogen. The resection specimens were formalin fixed and paraffin embedded for routine histopathological examination. Tumours staged as pT1 or pT4 and those with distant metastases were excluded from the study.

Sections of formalin-fixed, paraffin embedded tissue 5 mm thick were deparaffinized and rehydrated. Antigen retrieval was performed by means of a citric acid solution and microwave heating (15 min at 650 W). Endogenous peroxidase was blocked with 0.2% hydrogen peroxide. The E-cadherin-specific antibody HECD1 (Takara Biomedicals, Takara Shuzo, Japan) was used at room temperature at a dilution of 1:1000 with an incubation time of 1.5 h. Detection of bound antibody was carried out with the avidin-biotin complex peroxidase method (ABC Elite kit, Vector, Burlingame, Calif.) and was followed by staining with a peroxidase substrate (Sigma Fast DAB, Sigma, Deisenhofen, Germany). Replacement of the first antibody with phosphate-buffered saline served as a negative control.

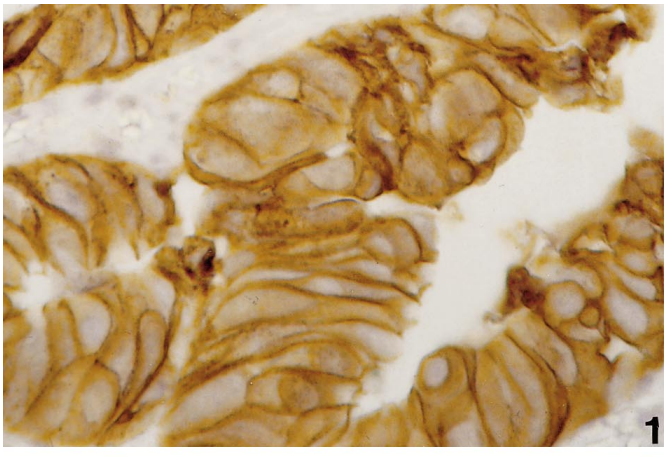
Staining was classified as normal when it was similar to nonmalignant mucosa as described previously (Fig. 1) [30], as weak in the case of barely visible staining (Fig. 2), as reduced for expression between normal and weak (Fig. 3) and as negative when there was no visible reaction (Fig. 4). In addition, the cellular distribution of staining was described as normal if it was membrane bound, and abnormal when it had an intracellular distribution (Fig. 3). The staining distribution pattern was scored as homoge-

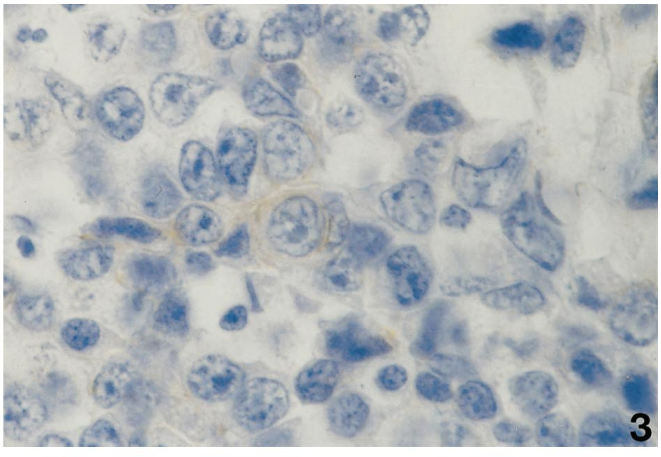
Fig. 1 An area of colon carcinoma showing E-cadherin expression with a normal intensity and membranous distribution. HECD-1 antibody (DAB development) and haematoxylin counterstain, original magnification ×252

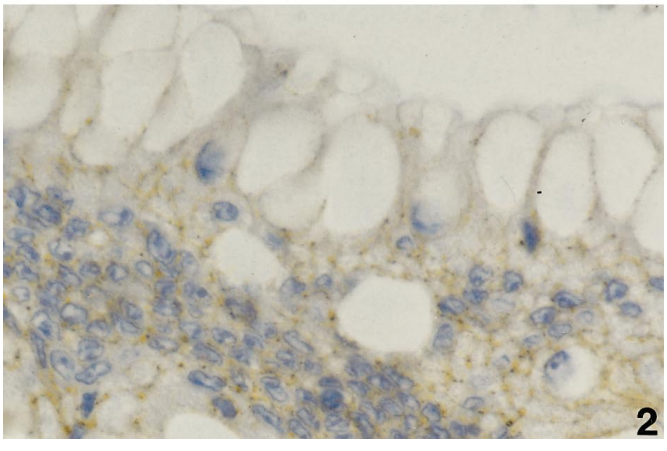
Fig. 2 A colon carcinoma showing E-cadherin expression with a reduced intensity. HECD-1 antibody (DAB development) and haematoxylin counterstain, original magnification ×252

Fig. 3 A colon carcinoma showing only focal, weak E-cadherin expression. HECD-1 antibody (DAB development) and haematoxylin counterstain, original magnification ×252

Fig. 4 A colon carcinoma case without E-cadherin expression. HECD-1 antibody (DAB development) and haematoxylin counterstain, original magnification ×252







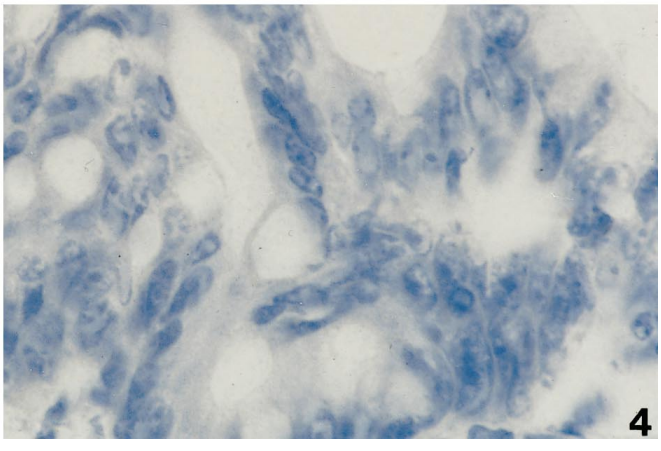


Table 1 E-Cadherin primers used for amplification and cycle dye-sequencing

	Primers used for amplification		Nucleotide position[a]	Length of PCR product	Primers used for cycle dye-sequencing	
	Name	Sequence (5′ to 3′)			Name	Sequence (5′ to 3′)
Forward	ATG	CCA TGG GCC CTG GAG CCG C	93–926	834 bp	rPre4	GCC AGG AGA GGA GTT GGG AA
Reverse	rEx6	CTG GAA GAG CAC CTT CCA TGA C			rEx5	ATC CAG AGG CTC TGT CAC CTT
Forward	Ex5	GGC AAA GAA GGC AAG GTT TTC	640–1780	1140 bp	rEx7	GGC GGC ATT GTA GGT GTT CAC
Reverse	rEx11	TGT GTA CGT GCT GTT CTT CAC			rEx9/1	AAA ATG CCA TCG TTG TTC ACT
					rEx9/2a	CAG CGT GGG AGG CTG TAT ACA C
Forward	Ex11	GGC TGG AGA TTA ATC CGG ACA C	1689–2836	1147 bp	rEx12	TTG GGT CGT TGT ACT GAA TGG TC
Reverse	r3prime/neu	CTC ATC TCA AGG GAA GGG AGC			Ex12/2 neu	GGT CAT AAA CAT CAT TGA TGC AGA C
					Ex15	GAC TTT GAC TTG AGC CAG CTG C

[a] Nucleotide positions refer to an E-cadherin sequence deposited in the EMBL/GenBank Data Libraries, accession no. Z13009

neous when the entire tumour area was stained, and heterogeneous when there were tumour areas without immunoreactivity. Eleven tumours (6 with weak and 5 with reduced staining) showed a heterogeneous pattern of staining. The remaining 10 (6 with normal and 4 with reduced staining) had a homogeneous pattern.

In the 8 cases with negative immunoreactivity with HEC-D1 antibody, tumour samples frozen at the time of surgical resection were used for standard guanidinium isothyiocyanate RNA extraction and cesium chloride centrifugation. A section from each of these samples was stained with haematoxylin and eosin to ensure that at least 70% of the sample consisted of tumour cells. The resulting mRNA (1 mg) was reverse-transcribed and then amplified by PCR in three overlapping segments spanning the entire coding region for E-cadherin (for cDNA primers see Table 1). The PCR-cycle conditions were: denaturation for 2 min at 94°C; annealing for 2 min at 58°C; and elongation for 2 min at 72°C (Taq polymerase and PCR buffer from Eurobio GmbH, Raunheim, Germany, and PCR buffer from Perkin-Elmer. Foster City, Calif.; thermal cycler from Biomed Labordiagnostik, Oberschleissheim, Germany). Each result was confirmed by repeating the PCR amplification and sequencing. The amplified cDNA fragments were purified by agarose gel electrophoresis and sequenced in a dye terminator cycle (thermal cycler 9600 from Perkin Elmer Cetus, USA) using rPre4, rEx5, rEx7, rEx9/1, Exon 9/2a, rEx12, Ex12/2/neu, Ex15 as internal primers (Table 1).

Purification of the amplified product was performed with Centri-Sep columns (registered trademark of Princeton Separations, USA) in order to eliminate nonincorporated dye terminators before determination of the sequence with an ABI Prism 377 Sequencer (trademark of Applied Biosystems, Perkin Elmer, USA). The sequencer settings were adjusted according to the Users' Manual, Revision A, January 1995 by Perkin Elmer, USA.

After discharge, the patients were examined by their general practitioner at defined intervals (every 6 months in the first 2 postoperative years followed by an annual examination). Survival data were calculated according to Kaplan and Meier [13]. Statistics were calculated using the log-rank test with a significance limit of $P<0.05$. Owing to the small number of patients, we did not perform multivariate analysis. Statistical analysis was performed with BMDP Statistical software, 1990 version (Cork, Ireland).

Results

Tissue sections from the 29 cases were examined using HEC-D1 as the E-cadherin-specific antibody. All specimens included nonmalignant mucosa as well as neoplastic areas, as an internal control of the immunoreactivity for each case. In all cases, the nonmalignant colonic mucosa showed homogeneous staining along the intercellular borders of the epithelial cells. In the corresponding malignant mucosa, three staining patterns with regard to intensity, cellular distribution and tissue distribution of the staining reaction were observed. Staining intensity similar to that in apparently normal mucosa was found in 6 tumours (21%) and was categorized as normal. Nine tumours (31%) had clearly visible staining which was less than in the surrounding normal mucosa, which was categorized as reduced. Six (21%) cases showed minimal staining and 8 tumours (28%) had no detectable E-cadherin immunoreactivity (Table 2). According to the staining pattern, 4 cases (13.7%) had a normal staining reaction along the intercellular borders, with the remaining 17 cases (58.6%) having scattered cytoplasmic staining with diminished localization of E-cadherin at the cell membrane. In 10 tumours cases (34%) the entire tumour

Table 2 Results of staining for HEC-D1 antibody in colon carcinoma. Staining is compared with staining of normal mucosa cells in the same histopathological section. Tumor grading according to WHO criteria

Staining intensity	*n*	Intracellular distribution	*n*	Intratumoral distribution	*n*
Normal intensity/ grading	6 (21%) G2 *n*=3 G3 *n*=3	membrane bound/ grading	4 (14%) G2 *n*=3 G3 *n*=1	Tumor homogeneous positive	10 (34%)
Reduced intensity/ grading	9 (31%) G2 *n*=6 G3 *n*=3	membrane bound and cytoplasmic/ grading	13 (44%) G2 *n*=7 G3 *n*=6	Tumor heterogeneous positive	11 (38%)
Minimal intensity/ grading	6 (21%) G2 *n*=5 G3 *n*=1	cytoplasmic only/ grading	4 (14%) G2 *n*=4 G3 *n*=0		
No staining observed/ grading	8 (28%) G2 *n*=3 G3 *n*=5	no staining observed/ grading	8 (28%) G2 *n*=3 G3 *n*=5	Tumor homogeneous negative	8 (28%)
Total number	29 (100%)		29 (100%)		29 (100%)

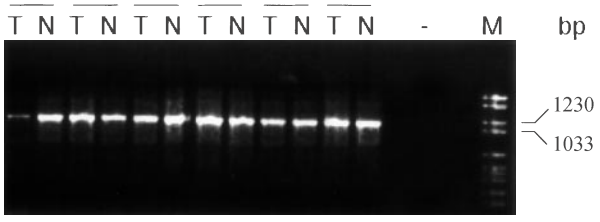


Fig. 5 A representative agarose gel showing PCR amplified E-cadherin cDNA fragments from six patients. Amplification products from non-tumours (*N*) and tumour (*T*) tissue are shown for each patient. The primer pair Ex5/Ex11 was used for PCR, resulting in amplification of a 1140-base-pair (bp) portion spanning exons 5–11. Sequence analysis of the tumour-associated PCR amplification products revealed no structural mutations. The sizes of the molecular weight markers (*M*), are indicated in base pairs. The lane labelled with the *minus sign* is the negative control

area showed immunoreactivity, which was classified as homogeneous in contradistinction to the remaining 11 (38%) cases, which had scattered tumour areas without staining (a heterogeneous pattern). There was no correlation between E-cadherin reactivity and standard prognostic factors such as tumour grade and stage.

To determine whether in the 8 cases without expression of E-cadherin with the HEC-D1 monoclonal antibody the pattern was due to E-cadherin gene alterations, we sequenced the E-cadherin cDNA reverse transcribed and amplified from these cases. Wild-type E-cadherin sequences without any detectable structural mutations were found in all 8 patients lacking reactivity with HEC-D1 antibody. Figure 5 shows a sequencing gel in a case with negative staining for E-cadherin; no mutation was identified.

Most (26, or 90%) of the patients had complete clinical follow-up and were available for analysis. One patient was lost to follow-up after 50 months, and 2 after 35 months. After a median follow-up time of 43 months (range: 25–72 months), 8 deaths were observed, which were due to colon carcinoma (liver metastasis or peritoneal carcinosis). The 5-year survival rate was 72%. The standard prognostic factors such as nodal involvement and tumour grade [8] showed a correlation with survival, but owing to the small number of cases this did not reach statistical significance (log rank test P=0.06 for grade; P=0.07 for nodal metastasis). We found that cases with absent or weak E-cadherin expression had better survival than patients with a normal staining pattern if E-cadherin staining was analysed independently of other factors, such as nodal involvement and grade. Cases with normal or reduced E-cadherin expression had a statistically significantly poorer survival than cases with little or no expression (in univariate analysis: log rank test P=0.05). Among the cases with normal or reduced E-cadherin expression 6 had nodal metastases, as against only 3 of the patients whose tumours showed weak E-cadherin expression or none at all.

Discussion

The role of cell adhesion in cellular differentiation and tissue organization and its breakdown in the development of carcinoma is of growing importance for our understanding of the invasive and metastatic potential of malignant neoplasms. Cadherins are a major component of the cell-cell interaction system, which is linked to the cytoplasmic actin skeleton. In epithelial tissues, E-cadherin is normally distributed along the cellular membrane but not on luminal cell surfaces [30]. Neoplastic epithelial cells of most organs show alterations of E-cadherin expression, especially cells of carcinomas of the head and neck [29], bladder [20], breast [12] and cervix uteri [35]. A significant reduction in E-cadherin expression has been observed in poorly versus well-differentiated ductal carcinoma in situ (DCIS) of the breast [10], which indicates that its loss is probably an early event in the development of ductal breast carcinoma. The data concerning colorectal carcinoma and E-cadherin expression are much less conclusive. Other investigators have found reduced or absent expression of E-cadherin in cases of co-

Table 3 Results of staining for HEC-D1 antibody in colon carcinoma in relation to histopathological stage (Staining is compared to staining of normal mucosa cells at the same histopathological section no staining: Ø; minimal intensity: +, reduced intensity: ++; normal intensity: +++ Tumor grading according to WHO criteria)

Case no.	Sex	Age	Segment of Colon	Dukes classification	pTNM stage	Grade	Histological subtype (WHO)	Immuno-staining intensity
1	Female	82	Ascending	B	pT3N0	3	Adenocarcinoma	++
2	Female	57	Ascending	B	pT3N0	2	Adenocarcinoma	+
3	Female	83	Ascending	C	pT3N2	3	Adenocarcinoma	++
4	Male	40	Ascending	B	pT3N0	3	Adenocarcinoma	Ø
5	Male	54	Sigmoid	B	pT3N0	3	Adenocarcinoma	++
6	Female	70	Ascending	C	pT3N1	2	Adenocarcinoma	++
7	Female	46	Transverse	B	pT3N0	2	Adenocarcinoma	+
8	Male	57	Descending	B	pT3N0	2	Adenocarcinoma	++
9	Female	60	Descending	C	pT3N2	3	Adenocarcinoma	+
10	Female	63	Sigmoid	C	pT3N1	3	Adenocarcinoma	+++
11	Female	61	Descending	B	pT3N1	3	Adenocarcinoma	+++
12	Female	54	Descending	B	pT3N0	2	Adenocarcinoma	Ø
13	Male	59	Descending	B	pT3N0	3	Mucinous adeno.	Ø
14	Female	52	Descending	A	pT2N0	2	Adenocarcinoma	++
15	Male	66	Sigmoid	B	pT3N0	3	Adenocarcinoma	Ø
16	Female	72	Ascending	B	pT3N0	3	Adenocarcinoma	Ø
17	Male	84	Ascending	B	pT3N0	2	Adenocarcinoma	Ø
18	Female	72	Cecum	B	pT3N0	2	Adenocarcinoma	Ø
19	Male	48	Ascending	B	pT3N0	2	Mucinous adeno.	+
20	Female	48	Sigmoid	B	pT3N0	2	Mucinous adeno.	++
21	Male	49	Transverse	B	pT3N0	3	Adenocarcinoma	Ø
22	Female	75	Cecum	B	pT3N0	2	Adenocarcinoma	++
23	Male	63	Ascending	B	pT3N0	2	Adenocarcinoma	+
24	Female	87	Sigmoid	C	pT3N1	2	Adenocarcinoma	+
25	Male	53	Sigmoid	C	pT3N2	2	Adenocarcinoma	+++
26	Female	82	Sigmoid	C	pT3N1	2	Adenocarcinoma	++
27	Male	70	Transverse	B	pT3N0	3	Adenocarcinoma	+++
28	Male	44	Ascending	B	pT3N0	2	Adenocarcinoma	+++
29	Male	85	Ascending	C	pT3N1	2	Mucinous adeno.	+++

lorectal carcinoma, as in our study, but were not able to correlate this with the invasive or metastatic potential of the tumours or with prognosis [17, 18, 23, 34].

In our series, only 4 tumours (13.7%) showed a staining pattern similar to that at apparently normal mucosa, all others (n=25) having either reduced expression or an abnormal, cytoplasmic E-cadherin distribution. The phenomenon of cytoplasmic E-cadherin immunoreactivity, which was seen in 17 (58%) of our tumours, has previously been described as correlated with the degree of dysplasia in cervical intraepithelial neoplasia and with differentiation of the tumour in pancreatic carcinoma [35]. Among the 17 moderately differentiated (G2) tumours we found cytoplasmic staining in 12 cases, whereas 3 tumours had normal, membrane-bound E-cadherin expression. Three tumours in the G2 group showed no membrane-bound E-cadherin expression. The 12 poorly differentiated (G3) tumours showed cytoplasmic expression in 6 cases, membrane-bound staining in 1 case and absent E-cadherin expression in 5 tumours (Table 2). Cytoplasmic staining has been interpreted as either protein on its way to the cell surface or as an artefact [35]. Since artefacts would also be seen in mucosa with a normal appearance, it seems more likely that cytoplasmic staining is a reflection of defects in the transfer or integration of bound E-cadherin to the cell membrane, or the result of an abnormal association with cytoplasmic proteins.

Reduced or absent immunoreactivity for E-cadherin correlated with a better survival in the Kaplan-Meier survival analysis. This surprising result might be due to the fact that in this subgroup the cases with nodal metastasis and less differentiated tumours were underrepresented compared with the group of E-cadherin immunoreactive cases. Among the cases with minimal or absent E-cadherin expression, 14 (80%) were free of nodal metastases and 8 were classified as G2 tumours compared with only 6 G3 tumours. More moderately than poorly differentiated tumours expressed E-cadherin (9 vs 6 cases). The unequal distribution between the two groups, particularly with respect to nodal metastases, might explain the unexpected correlation of abnormal E-cadherin expression with a better clinical course and should be re-examined in a larger number of patients with equal distributions of standard prognostic factors. In addition, these findings suggest that immunohistochemically detected E-cadherin protein expression is of minor importance compared with conventional prognostic factors, such as stage and grade, as far as its contribution to invasive and metastatic potential in colon cancer is concerned.

Genomic alteration of E-cadherin in cancer has been reported previously for gastric carcinoma [25]. Somatic alteration of E-cadherin messenger RNA has been identified in 50% of diffuse-type gastric carcinomas. In addition E-cadherin has been found to be mutated in a high

proportion (4 of 7 and 5 of 9 cases) of invasive lobular breast carcinomas [2]. In contrast to these results, our analysis of colon carcinoma revealed no E-cadherin mutations. The discrepancy between the lack of immunohistochemical staining and the apparently normal E-cadherin mRNA molecules might be explained by regulatory defects. A possible down-regulation of a E-cadherin promoter could be a key to a loss of E-cadherin expression leading to reduced cell integrity in these cases [3]. Regions close to the E-cadherin gene have been found to induce specific transcription factors and, in some dedifferentiated cells, these fragments are deleted. Further, E-pal, a specific sequence which binds transcription factors, has been described and appears to play a critical part in E-cadherin gene transcription [11]. A change of steric configuration in the region of the E-cadherin gene due to the methylation of CpG sites in the promoter region and a change in the chromatin ultrastructure of the promoter region have also been suggested as possible explanations for down-regulation of E-cadherin expression. This idea is supported by the observation that cells without E-cadherin expression tend to show DNA hypermethylation [37].

Our study illustrates that a mutation in the expressed DNA of the E-cadherin gene is only one of several potential mechanisms that might disturb the expression or function of E-cadherin in epithelial tumours. Carcinomas with a more infiltrative phenotype, such as diffuse gastric carcinoma and invasive lobular carcinoma of the breast, show a number of E-cadherin mRNA alterations, whereas tumours with an organized phenotype, including colon carcinoma and ductal carcinoma of the breast, are apparently more likely to suffer from a regulatory deficit of E-cadherin protein expression [14]. The lack of correlation between E-cadherin expression and standard prognostic factors in colorectal carcinoma is evidence that the interaction between E-cadherin protein expression and colorectal tumour cell behaviour is complicated. Since tumour cell invasion and metastasis are complex processes it is likely that other abnormalities of cell adhesion molecules, such as DCC, CD44 and nm23, as well as the cadherin-associated catenins, could outweigh the potential beneficial effect of normal E-cadherin expression [5, 16, 26, 28, 31]. Further studies are warranted to clarify the complex interaction of these molecules and their impact on malignant progression, and in particular on invasive and metastatic potential.

Acknowledgement This work was supported by the Wilhelm Sander Foundation (grant no. 94.078.1).

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