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Changes in E-cadherin immunoreactivity in the adenoma-carcinoma sequence of the large bowel

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Abstract We have used an avidin-biotin immunoperoxidase technique to localise epithelial cadherin (E-cadherin), a calcium-dependent cell-cell adhesion molecule, in 107 paraffin-embedded sections from 93 patients consisting of 24 with colorectal adenoma, 55 with rectal carcinoma and 14 with liver metastases. The corresponding primary colorectal tumours were also studied in these cases. E-cadherin was expressed by normal colorectal epithelial cells with typical membranous staining at the intercellular junctions. Loss of normal membranous E-cadherin expression and presence of cytoplasmic staining were found frequently in adenomas larger than 1 cm ($P<0.01$), with high grade dysplasia and villous histology ($P<0.01$). In primary rectal cancers, loss of membranous expression correlated with high tumour grade. No correlation was seen with Dukes and Jass stage, local extramural spread and 5-year recurrence rate. Complete loss of membranous E-cadherin immunoreactivity was seen in 7/14 (50%) liver metastases in which 6/7 (86%) showed intense membranous E-cadherin immunoreactivity in the corresponding primary tumour. Our data indicate that changes in E-cadherin immunoreactivity and cellular localisation correlate with size, severe dysplasia in adenomas and tumour grade in carcinomas. However, there seems to be no correlation between loss of membranous E-cadherin immunoreactivity and the invasive and metastatic potential of the carcinomas.

Key words E-cadherin · Colon · Adenoma · Carcinoma · Tumour grade

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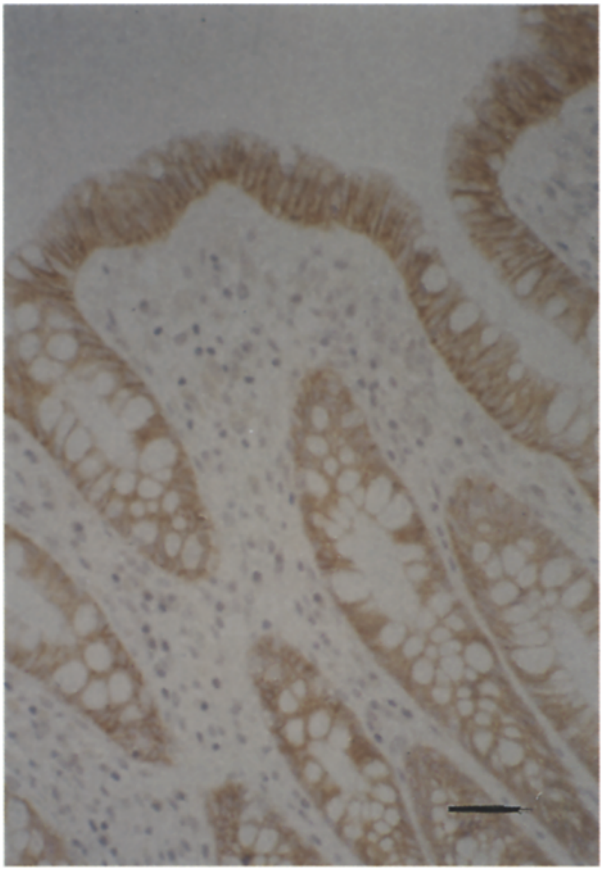
Introduction

Colorectal tumourigenesis is a result of multiple genetic alteration causing uncontrolled proliferation, tissue invasion and metastasis [2, 6]. Most colorectal carcinomas seem to arise from adenomas which are classified according to the histological structure as tubular, tubulovillous and villous [9]. Dysplasia, large size and villous histology are the major characteristics of the adenomas with malignant potential [16]

Alterations in the function and expression of adhesion receptors which mediate cell-cell and cell-substrate interactions have been shown to determine the phenotype and malignant behaviour of colorectal cancer [19]. E-cadherin (also named L-CAM, uvomorulin, Arc-1 and cell-CAM 120/80), in particular, is the prime mediator of epithelial cell-cell adhesion via calcium dependent, homotypic interactions, that is to say a molecule on one cell binds to cadherin molecules of the same type on another cell [29]. E-cadherin participates in the formation of adherens junctions and its function is in part regulated via the carboxy terminal intercellular domain by α, β and γ catenins which interact with cytoskeletal elements of the cells which they bind [18]. E-cadherin is required for the induction and maintenance of normal epithelial integrity [29]. In vitro, loss of E-cadherin has been associated with an invasive and poorly differentiated phenotype of colon carcinoma cells [22]. Transfection of E-cadherin cDNA into a poorly differentiated human colon carcinoma cell line increases cell polarity and intercellular cohesion, and inhibits invasion in vitro [12] A number of clinico-pathological studies have demonstrated that loss of E-cadherin expression is commonly associated with high grade and advanced stage in a variety of malignancies including breast carcinoma, prostatic adenocarcinoma, bladder tumours and squamous carcinoma of the head and neck [20, 30]. In colorectal cancer, however, alterations in E-cadherin expression have not been found to correlate with its metastatic potential consistently [4, 11, 17, 31, 32]. However, in previous studies no paired samples of liver metastases and corresponding primary tumours



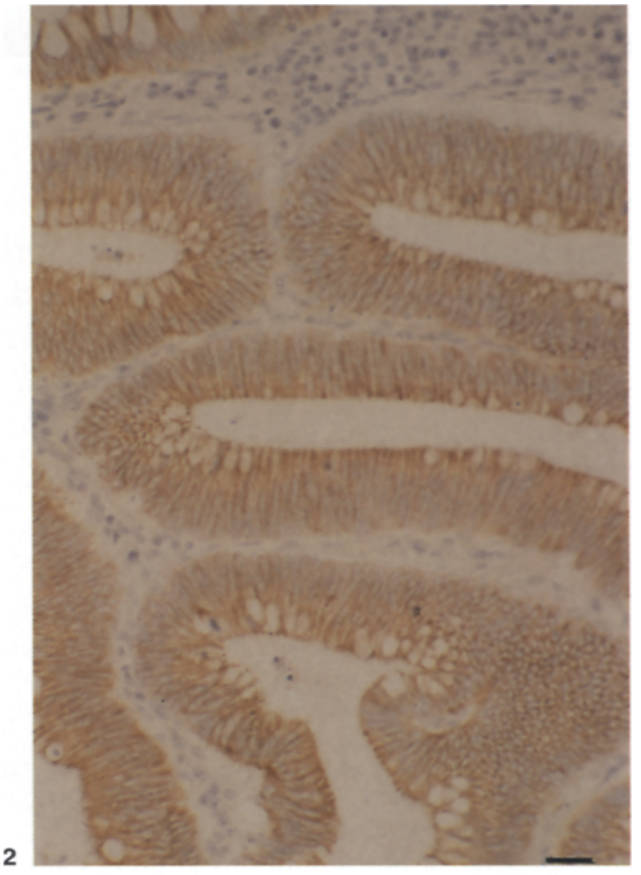


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Fig. 1 Avidin-biotin indirect immunoperoxidase staining of normal colorectal epithelial cells showing membranous E-cadherin immunoreactivity (avidin-biotin indirect immunoperoxidase staining, bar=25 μm, magnification ×200)

from the same patient were examined nor were correlations with prognosis and recurrence rate examined.

To this end, we have studied E-cadherin immunoreactivity and its cellular localisation in microwave-treated, paraffin-embedded sections from a series of 24 colorectal adenomas, 55 rectal carcinomas and 14 liver metastases with the corresponding primary tumours.

Materials and methods

One hundred and seven samples from 93 patients were included in this study. Twenty-four colorectal adenomas and 55 surgically resected primary rectal carcinomas were obtained from the archival material of St Mark's Hospital, London (UK). All patients with rectal cancer had a complete 5 year follow up and a known pattern of recurrence. In addition, 14 liver metastases were obtained from King's College Hospital and Hammersmith Hospital, London (UK). Paraffin blocks from the 14 corresponding primary tumours were obtained from a number of hospitals throughout United

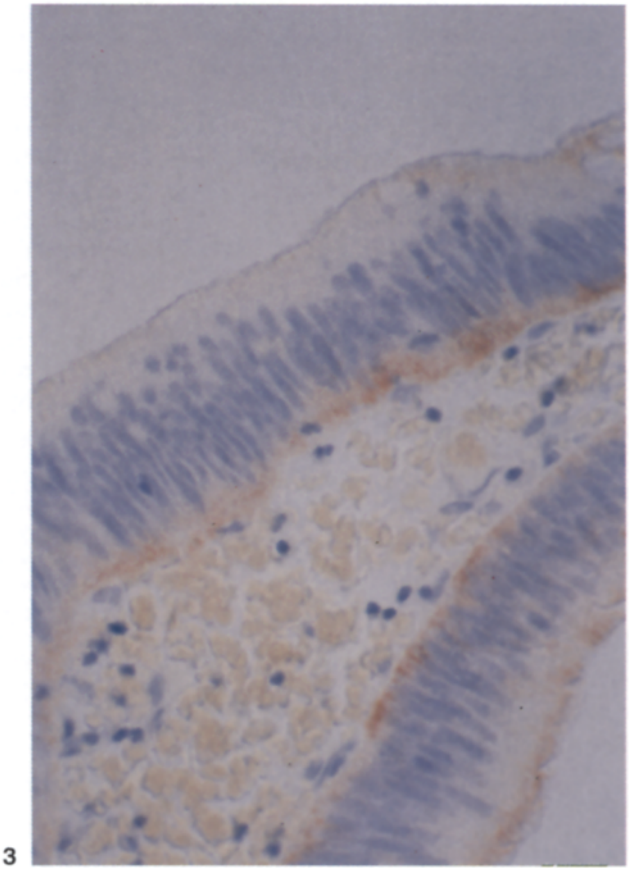

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Fig. 2 Preserved E-cadherin immunoreactivity in a tubular adenoma (avidin-biotin indirect immunoperoxidase staining, bar=25 μm, magnification ×200)

Fig. 3 Cytoplasmic staining and loss of membranous E-cadherin immunoreactivity in a villous adenoma (avidin-biotin indirect immunoperoxidase staining, bar=50 μm, magnification ×400)

Kingdom. Serial sections were stained with haematoxylin and eosin and used for histological classification of the lesions identified. Tumours were graded and classified according to Dukes [5] and Jass classifications [10] without knowledge of the staining results. Local extramural spread of tumour beyond the muscularis propria was described as absent, minimal, slight or extensive, based on a subjective assessment [14].

The avidin-biotin indirect immunoperoxidase method was employed using the anti-E-cadherin HECD-1 (Human Epithelial Cadherin-1) mouse monoclonal antibody as undiluted culture supernatant. HECD-1 has been characterised previously and its specificity is described elsewhere [26]. To enhance E-cadherin immunohistochemistry in formalin-fixed, paraffin-embedded tissues, sections were treated with an antigen retrieval solution in a microwave oven, according to our previously described methods [8]. Briefly the slides were submerged in 0.01 M citrate buffer at pH 6.0 and are heated in a 700 W microwave on full power for 5×2 min cycles pausing to ensure there is no fluid loss due to evaporation. Slides were then rinsed in phosphate buffered saline (×3) after each stage. Fifty microliters of anti-E-cadherin HECD-1 primary antibody was then added to the section and incubated overnight at 4° C. An avidin-biotin complex immunoperoxidase technique was utilised to amplify epitope recognition (ABC kit, Dako, High Wycombe, UK) and subsequent staining was achieved by 50 µl diaminobenzidine solution, at a concentration of 0.3 mg/ml (Dako). The slides were then washed and mounted for microscopic examination. Positive control tissue sections known to be of a homogeneous phenotype were used to ensure accurate and reproducible staining and included normal small and large intestinal mucosa. Normal colorectal epithelial cells or normal hepatocytes present in the primary or metastatic tumour sections respectively were also used as internal positive controls for E-cadherin staining which was normally seen at the cell-cell borders. Negative controls were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins.

Slides were scored by assessing the proportion of stained cells, intensity of staining relative to the adjacent normal mucosa and site of staining (membrane or cytoplasmic) by three independent observers (GG, OK, MP) as previously described [8].

Fisher's exact test was used to compare E-cadherin immunoreactivity with the histopathological data. Spearman test was used to correlate loss of membranous expression with presence of cytoplasmic staining. Values of P less than 0.05 were considered significant.

Results

Expression of E-cadherin in normal colorectal epithelium

Membranous E-cadherin staining was localised uniformly at the intercellular borders of histologically normal colorectal epithelial cells present in the tumour specimens. No cytoplasmic immunoreactivity was seen (Fig. 1).

Expression of E-cadherin in colorectal adenomas

Membrane staining of intensity equal to the adjacent normal mucosa was seen in 18/24 (75%) colorectal adenomas (Fig. 2) whereas cytoplasmic staining was observed in 10 cases (40%). The presence of cytoplasmic E-cadherin immunoreactivity was statistically associated with loss of membranous expression (Spearman correlation $P<0.002$). This abnormal pattern was more frequently seen in large adenomas (more than 1 cm in diameter) ($P<0.01$) with high grade dysplasia ($P<0.05$) and villous histology ($P<0.05$) (Table 1) (Fig. 3).

Expression of E-cadherin in rectal carcinomas

E-cadherin immunoreactivity was present in 42/55 (76%) (Table 2). Staining was focal (<30% of positive cells) in 11 (20%) tumours and diffuse in 31 (56%) (Fig. 4). Thirty-one percent (9/24) of tumours with negative or focal staining were poorly differentiated compared to 10% (3/31) of tumours with a diffuse staining ($P<0.05$). Preserved membranous E-cadherin immunolocalisation

Table 1 E-cadherin immunoreactivity in colorectal adenomas

	Total	Pattern		Membrane		Cytoplasm	
		<30%	>30%	+	−	+	−
Total							
n	24	7	17	18	6	10	14
(%)	(100)	(29)	(71)	(75)	(25)	(42)	(58)
Histology							
Tubular							
n	13	4	9	11	2	3	10
(%)	(54)	(31)	(69)	(85)	(15)	(23)	(77)
TV/V							
n	11	3	8	7	4	7	4
(%)	(46)	(27)	(73)	(64)	(36)	(64)	(36)
Dysplasia							
Mild							
n	13	3	10	10	3	3	10
%	(54)	(23)	(77)	(77)	(23)	(23)	(77)
Moderate/ severe							
n	11	4	7	8	3	6	5
%	(46)	(36)	(64)	(73)	(27)	(55)	(45)
Size							
>1 cm							
n	16	5	11	12	4	8	8
%	(67)	(31)	(69)	(75)	(25)	(50)	(50)
<1 cm							
n	8	2	6	6	2	2	6
%	(33)	(25)	(75)	(75)	(25)	(25)	(75)

Table 2 Correlation of E-cadherin immunoreactivity with tumour grade in patients with rectal cancer

	Total	Pattern			Membrane		Cytoplasm	
		Neg	<30%	>30%	+	−	+	−
Total								
n	55	13	11	31	17	38	24	31
(%)	(100)	(24)	(20)	(56)	(31)	(69)	(44)	(56)
Grade								
Well								
n	2	0	0	2	2	0	0	2
(%)	(4)	(0)	(0)	(100)	(100)	(0)	(0)	(100)
Moderate								
n	41	8	7	26	15	26	20	21
(%)	(74)	(20)	(17)	(63)	(37)	(63)	(49)	(51)
Poor								
n	12	5	4	3	0	12	4	8
(%)	(22)	(42)	(33)	(25)	(0)	(100)	(33)	(67)

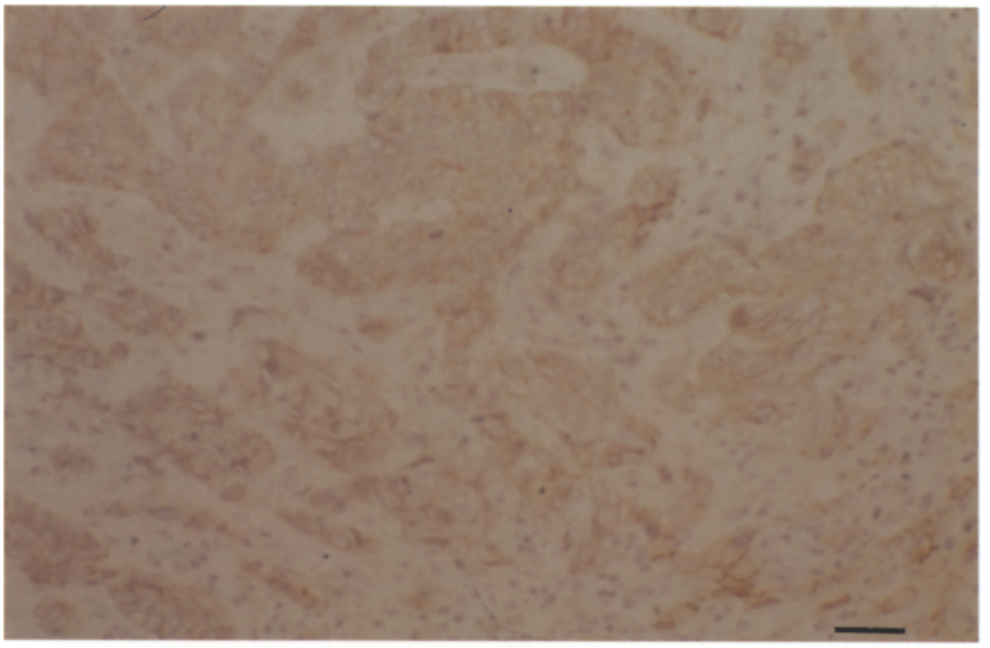

Fig. 4 Heterogenous E-cadherin immunoreactivity in a moderately differentiated rectal adenocarcinoma (avidin-biotin indirect immunoperoxidase staining, bar=25 μm, magnification ×200)

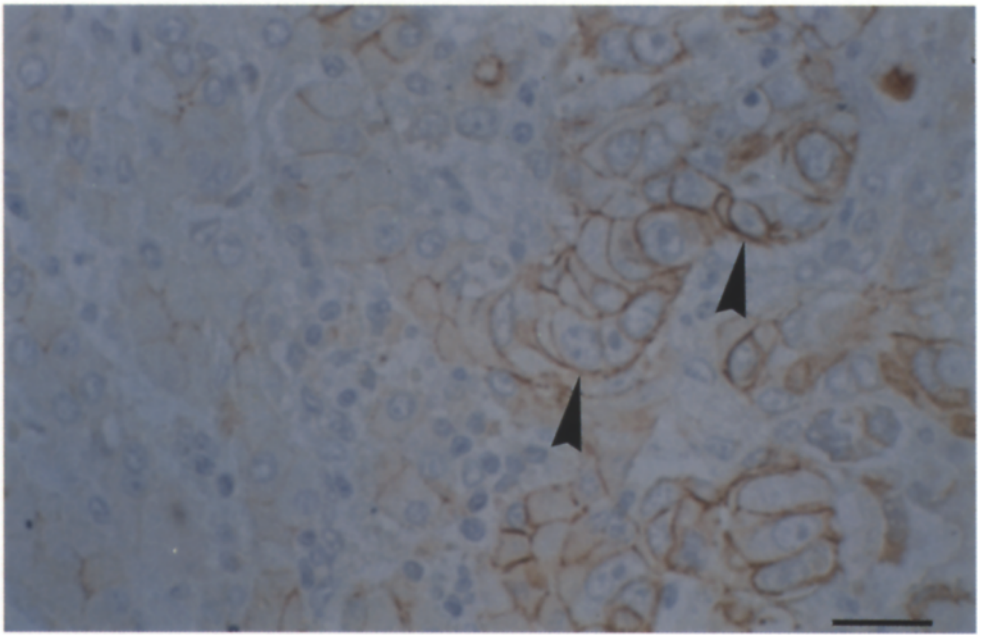

Fig. 5 Preserved E-cadherin immunoreactivity (*arrowheads*) in a liver metastasis from a moderately differentiated colorectal carcinoma (avidin-biotin indirect immunoperoxidase staining, bar=50 μm, magnification ×400)

Table 3 Correlation of membranous E-cadherin immunoreactivity with stage in patients with rectal cancer

	Total *n* (%)	Positive *n* (%)	Negative *n* (%)
Total	55 (100	17 (31)	38 (69)
Dukes' A	8 (14)	3 (37)	5 (63)
Dukes' B	20 (36)	6 (30)	14 (70)
Dukes' C	27 (49)	8 (30)	19 (70)
Jass 1	11 (20)	5 (45)	6 (55)
Jass 2	14 (25)	4 (29)	10 (71)
Jass 3	18 (33)	3 (17)	15 (83)
Jass 4	12 (22)	5 (42)	7 (58)

was seen more frequently in well or moderately differentiated tumours than in poorly differentiated tumours ($P<0.01$). No correlation was seen between loss of membranous E-cadherin expression and advanced Dukes and Jass stages (Table 3), local extramural spread (Table 4) and 5-year recurrence rate (Table 5).

Expression of E-cadherin in liver metastases and the corresponding primary colorectal tumours

Fourteen liver metastases and the corresponding primary colorectal tumours were studied (Table 6). Seven out of 14 (50%) liver deposits exhibited preserved E-cadherin immunoreactivity on the cell membrane (Fig. 5). Com-

Table 4 Correlation of membranous E-cadherin immunoreactivity with extramural spread in patients with rectal cancer

	Total *n* (%)	Positive *n* (%)	Negative *n* (%)
Total	55 (100)	17 (31)	38 (69)
Absent	12 (22)	5 (42)	7 (58)
Minimal	13 (24)	5 (38)	8 (61)
Slight	17 (31)	3 (18)	14 (82)
Extensive	13 (23)	4 (31)	9 (69)

Table 5 Correlation of membranous E-cadherin immunoreactivity with 5 year recurrence rate in patients with rectal cancer

	Total *n* (%)	Positive *n* (%)	Negative *n* (%)
All patients	55 (100)	17 (31)	38 (69)
Non-recurrence	24 (44)	7 (29)	17 (71)
Recurrence	31 (56)	10 (32)	21 (68)

plete loss of membranous E-cadherin was seen in 7/14 (50%) (JH, DS, JF, AH, BS, WH, SB) liver metastases of which 6/7 showed intense membranous immunoreactivity in the corresponding primary tumour.

Discussion

In this study we have investigated the expression of immunoreactive E-cadherin in colorectal adenomas, carcinomas and liver metastases using a microwave antigen retrieval method in formalin-fixed, paraffin-embedded tissue sections. The application of this method to archival material has several advantages as retrospective studies can be performed easily. In addition paraffin sections exhibit better morphological preservation when compared with frozen sections. Moll et al. [13] as well as our own group [8, 23] have demonstrated clearly that microwave antigen retrieval is a reliable technique to detect E-cadherin protein in formalin-fixed, paraffin-embedded tissues.

In this study normal colorectal epithelial cells showed membranous expression of E-cadherin protein at the cell-cell borders which reflects the normal localisation of an intercellular adhesion molecule. As the molecule has to be present at the cell surface to permit homotypic adhesion, cytoplasmic E-cadherin is by definition non-functional. Abnormal staining patterns (negative, heterogeneous or cytoplasmic only) were seen frequently in adenomas as well as adenocarcinomas of the large bowel, as previously shown [4, 11, 17, 31, 32]. As in other human tumours the abnormal E-cadherin staining pattern is likely to reflect reductions or loss of E-cadherin-mediated adhesion. There is evidence to suggest that this abnormal pattern of E-cadherin expression is not an accessory finding in colorectal tumours as it has been demonstrated that a number of cell lines dedifferentiate in vitro when treated with anti-E-cadherin antibodies [1, 22] and become more polarised and differentiated when transfected with E-cadherin cDNA [7, 12]. Since E-cadherin is known to be the prime mediator of intercellular cohesion and epithelial tissue integrity, our data indicate that alterations in E-cadherin expression and function occur in pre-malignant lesions and, therefore, may play a role in the progression of adenomas with malignant potential.

E-cadherin may be detectable in the cytoplasm for a variety of theoretical reasons including an increased production rate or a failure to translocate or to anchor in the membrane. Alterations in α-, β- and γ-catenins which link the cadherin molecule to the actin cytoskeleton may underlie the abnormal cellular localisation of E-cadherin in adenomas. In particular, α and β catenins have been shown to bind the APC gene product [25, 28] which is frequently truncated in colorectal adenomas [24]. Recently, wild-type but not mutant APC has been shown to associate and promote the assembly of microtubules which influence the distribution of actin filaments and the cytoskeleton organisation in general [15, 27]. It is tempting to speculate that APC mutations may affect the stabilisation and/or functional state of the E-cadherin/catenin complex and, perhaps, other signalling molecules linked to the cytoskeleton. Indeed, both APC mutations [3] and reduced E-cadherin and integrin ex-

Table 6 Membrane or cytoplasmic E-cadherin immunoreactivity in primary colorectal tumours and liver metastasis (*M*, membrane; *C*, cytoplasm; *O*, negative; *NA*, not available

	Primary tumours			Liver metastasis		Overall survival
	Dukes'	Grade	E-cadherin	Grade	E-cadherin	
JF	A	mod	M+C	well	M	2 months
BVH	B	mod	M+C	mod	M	NA
JH	C	mod	C	mod	0	23 months
DS	B	mod	M	mod	0	2 months
GC	C	mod	M+C	mod	M	10 years
JF	C	mod	M	mod	0	18 months
AH	B	mod	M+C	mod	0	1 week
KJ	C	mod	C	well	M+C	2 years and 8 months
MK	C	well	M+C	mod	M	3 years and 7 months
CS	C	mod	M	mod	M	10 years
BS	C	mod	M+C	poor	0	2 years and 5 months
JW	C	mod	M+C	mod	M	NA
WH	B	mod	M+C	mod	C	8 months
SB	C	mod	M+C	mod	C	6 months

pression [21] seem to occur frequently in large adenomas with villous histology and high grade dysplasia.

Alterations in E-cadherin expression and cellular localisation in the primary tumours were also found to correlate significantly with tumour grade but not with the ability to metastasise to the liver. In our series in which for the first time a direct comparison of E-cadherin expression in paired samples of primary tumours and the corresponding liver metastases was made, membranous E-cadherin expression was seen in 50% of liver deposits. It is possible that a disregulation of intercellular adhesion may occur transiently at the time of invasion to allow the cells to detach from the primary site. However, re-expression of E-cadherin on the cell membrane may be required to allow the tumour cells to localise and re-establish at the distant site.

In conclusion, we have demonstrated that changes in E-cadherin immunoreactivity and cellular localisation occur in adenomas as well as carcinoma of the large bowel. Further studies are in progress to identify the mechanisms underlying the alterations in E-cadherin protein and its functional interaction with the catenin/APC complex.

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