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Short Communication

E-Cadherin Expression in Colorectal Cancer

An Immunocytochemical and In Situ *Hybridization Study*

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Expression of the epithelial-specific adhesion molecule E-cadherin has been assessed in paraffin-embedded tissue from a series of 72 colorectal carcinomas. Using immunocytochemistry and in situ hybridization it was found that E-cadherin expression was related inversely to tumor differentiation. Out of 44 well- and moderately differentiated tumors, 36 expressed good positivity, whereas 24 of 28 poorly differentiated tumors were E-cadherin-negative. Classification by Dukes stage revealed a highly significant difference (P << 0.001) between A and B (32 positive, four negative) and C1 and C2 (seven positive, 29 negative) stages in terms of immunoreactivity. Of the 32 lymph node metastases studied, 20 were negative for E-cadherin expression, as were seven of eight liver metastases. These results indicate that the down-regulation of E-cadherin levels in vivo is associated with the dedifferentiation, progression, and metastasis of colorectal cancer. (Am J Pathol 1993, 142:981–986)

Colorectal cancer spreads locally by direct invasion through the bowel wall into adjacent structures and by metastasis, through lymphatic and venous channels, to regional lymph nodes and distant organs.[1] For this process to occur, malignant cells initially must detach from the primary tumor mass and become motile.[2] The ability of malignant cells to modulate their intercellular cohesiveness is considered to be an early and pivotal event in tumor progression.[3]

E-cadherin (ECD) is a member of a family of transmembrane glycoproteins that mediate homotypic, calcium-dependent cell-cell adhesion in epithelial tissues.[4] Loss of ECD expression leads to the dissociation of cells from coherent tissues,[5,6] and several experimental studies have shown a causal relationship between down-regulation of ECD expression in tumor cells and the acquisition of an invasive phenotype.[7–10] A limited, but rapidly increasing, number of studies using immunostaining of different carcinomas has suggested there is an inverse correlation between ECD expression and tumor grade.[11–14] However, to date, these studies primarily have utilized immunofluorescence in frozen sections as a means of assessing the expression of ECD, which can lead to difficulties in the evaluation of tissue architecture. Consequently, we have investigated the expression of ECD in human colorectal adenocarcinomas and their metastases using paraffin-embedded archival material. Our results show that ECD expression is related closely to the stage and grade of this tumor type, with more aggressive cancers displaying marked reductions of this cell-cell adhesion molecule.

Material and Methods

Specimens

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas were obtained from archival material held in the Histopathology Department of St. Mark's

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Hospital, London. Equal numbers of lymph node–negative (36 Dukes A and B stage) and lymph node–positive (36 Dukes C1 and C2) tumors were selected consecutively, in chronological order, from the 4-year period between 1988 and 1991.

Immunostaining

Four-μ-thick paraffin sections were cut, attached to glass slides by melting the wax at 65 C, and then stored at room temperature. Sections were then dewaxed, hydrated, and treated with 0.1% chymotrypsin (Sigma Chemical Co., Poole, UK) and 0.1% calcium chloride at pH 7.8 for 15 to 20 minutes at 37 C. Following trypsinization, the sections were washed in water, incubated in 3% hydrogen peroxide (FSA Laboratory Supplies, Loughborough, UK) for 5 minutes and washed in phosphate-buffered saline, pH 7.5, for 5 minutes.

The primary antibody, DECMA 1 (Sigma Chemical Co., Poole, UK), a rat monoclonal antibody raised against murine E-cadherin,[5] was then applied at a dilution of 1 in 400 (in phosphate-buffered saline/1% fetal calf serum) for 60 minutes at room temperature. Immunostaining was performed using the Vectastain ABC kit specific for use with rat monoclonals (Vector Laboratories, Peterborough, UK). Final staining was developed with the Vector DAB substrate kit (Vector Laboratories, Peterborough, UK). All tumor sections included normal colorectal epithelium as an internal positive control. Negative controls consisted of consecutive sections in which the primary antibody was replaced with phosphate-buffered saline or a monoclonal antibody of the same species and IgG isotype (rat IgG1; Serotec, Oxford, UK).

In Situ *Hybridization*

An anti-sense riboprobe was prepared from a SmaI digest of a 386-base pair partial ECD complementary DNA (HC61) in a Bluescript SK vector (generously provided by Professor W. Birchmeier) by a T3 RNA polymerase, using ^{35}S-labeled UTP (Amersham, Aylesbury, UK). Hybridization to the sections was performed essentially as described by Senior et al.[15] As a control for any possible nonspecific binding of vector sequences within the ECD anti-sense riboprobe, we used an anti-sense probe to VLA-2 messenger RNA[16] in which 1083 bases (*Eco*RI to *Bgl*II) of the VLA-2 sequence were subcloned into the *Eco*RI and *Bam*HI sites of pBluescript II SK+. This construct yields identical riboprobe leader sequences to our ECD probe, but application of equal numbers of cpm of these probes to duplicate sections gave different distributions of autoradiographic grains. The ECD probe generally resulted in specific clustering of grains, whereas the VLA-2 probe rarely did so. In addition to this regular control we also examined five representative tumors in which RNAse treatment (100 μg/ml RNAse A at 37 C for one hour) and hybridization with a sense ECD riboprobe were used as further controls. The presence of hybridizable message in all tissue compartments was assessed by using a riboprobe, hβA-10, to detect β-actin messenger RNA. hβA-10 is the approximate 450-base pair fragment of the original clone pHFβA3′ut.[17]

Results

Clear and unequivocally strong ECD staining of normal colorectal epithelium was used in all sections as an internal positive control against which an assessment of ECD expression in tumor cells was made. In liver metastases, recognisable staining of normal bile duct epithelium was, wherever possible, also used as an internal positive control. The grade of the tumor and the presence of lymph node or liver metastases were confirmed by the pathologist (JPS) on consecutive hematoxylin and eosin–stained sections. ECD staining was assessed in the same area of the section as that used to determine tumor grade. The intensity of ECD staining in tumor cells was scored as ECD++ if equal to, or approaching, that of normal epithelium. In cases where the staining was clearly recognisable but weaker than normal epithelium, the tumor was evaluated as ECD+. Where expression was lost completely, the evaluation was scored as ECD−. ECD expression in lymph node and liver metastases was defined as ECD+ if present and as ECD− if no clearly recognisable staining was detected.

Figure 1a illustrates the typical pattern of staining observed in normal epithelium and Figure 1, b and c, the strong positivity in the majority of well-differentiated tumors with distinct intercellular localization of the stain. Figure 1d reveals the heterogeneity of expression in moderately differentiated tumors, whereas Figure 1, e and f, illustrate a negative poorly differentiated tumor and a positive lymph node deposit respectively. Figure 2b shows strong *in situ* hybridization positivity for E-cadherin messenger RNA in a well-differentiated tumor, whereas in a poorly differentiated tumor (Figure 2d), the signal is not discernible above background reactivity.

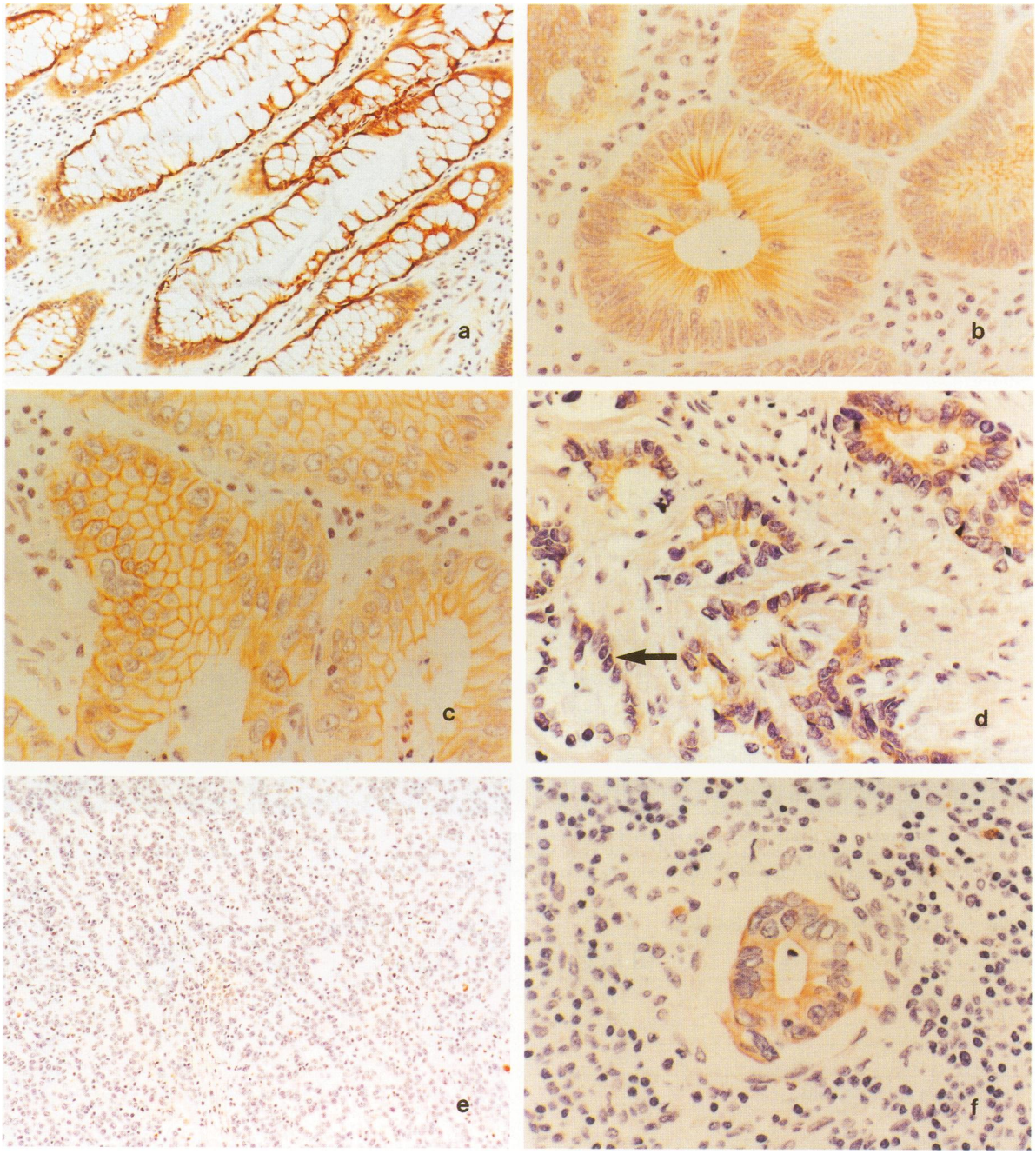

Figure 1. *All illustrations are under high power with immunoperoxidase staining.* **a:** *Normal colonic mucosa showing typical staining of epithelial cell membranes.* **b** *and* **c:** *Well-differentiated adenocarcinomas of the rectum revealing lateral membrane positivity* **(b)** *and intercellular honeycomb pattern similar to normal epithelium* **(c).** **d:** *Moderately differentiated tumor, illustrating heterogeneity of ECD expression. Loss of expression occurs (arrowed) in less well-differentiated areas.* **e:** *Complete negativity in a poorly differentiated adenocarcinoma.* **f:** *Lymph node metastasis revealing strong positivity.*

These results were representative of the analysis of 40 tumors by this technique. The use of RNAse and sense controls confirmed that ECD hybridization was indeed specific (results not shown). The results are summarized in Tables 1 to 3.

Discussion

That intercellular cohesion between malignant epithelial cells must be down-regulated as a prerequisite for invasive behavior was first proposed by

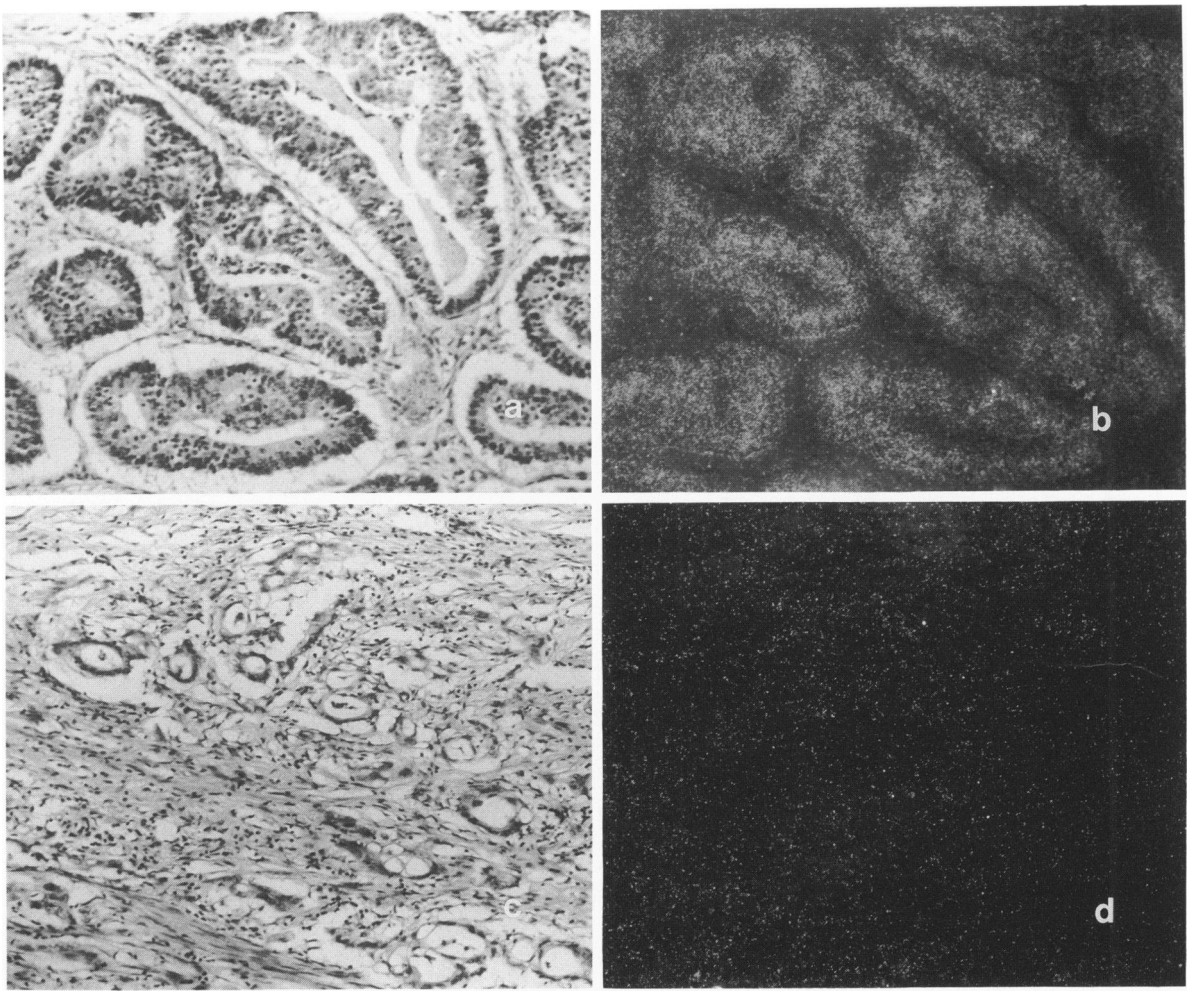

Figure 2. *All illustrations are under high power;* **(a)** *and* **(c)**, *hematoxylin and eosin.* **a** *and* **c**: *Bright-field views of moderately well-differentiated* **(a)** *and poorly differentiated* **(c)** *adenocarcinomas.* **b** *and* **d**: *Dark-field views of the same tumor areas showing strong signal for E-cadherin transcript in the differentiated tumor* **(b)** *and no reactivity in the poorly differentiated adenocarcinoma* **(d)**.

Table 1. *ECD Expression and Dukes Stage*

ECD expression	A & B	C1 & C2
ECD++/ECD+	32	7
ECD−	4	29
	36	36

Fisher exact test $P << 0.001$.

Table 2. *ECD Expression and Tumor Grade*

ECD expression	Well	Moderate	Poor
ECD++	7	4	0
ECD+	1	24	4
ECD−	0	8	24
	8	36	28

Coman and colleagues almost fifty years ago.[18,19] More recently, the elegant studies of Vogelstein and his group have provided an insight into the possible molecular basis of this phenomenon in colorectal carcinoma with the identification of the deleted in colon carcinoma (DCC) gene.[20] This gene, which encodes for a protein with strong sequence homology to neural cell adhesion molecule, is deleted allelically in 70% of colorectal carcinomas.[21] Additionally, anti-sense RNA technology has now yielded the first direct biological evidence supportive of the role of DCC in cell adhesion and as a tumor-suppressor gene.[22]

Another candidate molecule for maintaining the cohesiveness and epithelial integrity of colorectal cancers is E-cadherin.[4] We show here that ECD expression is related inversely to the grade and Dukes stage of the tumor and that, in general, metastatic deposits are ECD-negative. Broadly similar results have been documented in a number of other carcinomas[11–14] though these investigations have not used paraffin-embedded, archival material.

Table 3. *ECD Expression in Lymph Node Metastases*

Tumor Expression	Lymph node expression	
	ECD+	ECD−
ECD++/ECD+	3	3
ECD−	9	17
	12	20

Utilization of this material has facilitated the morphological analysis of the stained tissue and revealed, for example, that ECD expression in moderately differentiated tumors was heterogenous with high levels in well-formed tubules but less staining in areas of architectural atypia (Figure 1d). Expression and location of ECD protein, as indicated by immunostaining, correlated well with *in situ* hybridization analysis of messenger RNA transcripts (Figure 2).

Lymph node metastasis seemed to occur with greater frequency in cases where the primary tumor was ECD-negative (29 out of 36 such cancers) rather than when the primary lesion exhibited immunoreactivity for ECD (four out of 36 tumors). This pattern was reflected in the secondary deposits with 20 out of 32 metastases to the lymph nodes and seven of eight hepatic metastases failing to show positivity.

Interestingly, nine out of the 26 lymph node metastases arising from ECD-negative primary tumors exhibited strong positive staining (Table 3). This would seem to suggest that such secondary deposits derive from undetected areas of ECD positivity within the primary mass or that reemergence of ECD expression may occur at a different anatomical location. Such findings accord well with the possibility that the observed down-regulation of ECD expression may be a consequence of multiple mechanisms[23] rather than just those currently thought to be involved in the changes in DCC gene expression.[20] The ECD gene is located on chromosome 16q22.1,[24] and allelic loss of this region has been identified in a variety of epithelial tumors[25–27] consistent with the possibility that ECD functions in a tumor-suppressor role.[28] However no deletions or gross rearrangements of the gene were detected by Southern blotting of a number of ECD-negative human carcinoma cell lines.[7]

A previous report[29] has noted that ECD expression was weaker in tumors than in normal colorectal epithelium, though no association was made with tumor grade. Our study has extended these results and suggested there may be an application of ECD assessment as a prognostic indicator. The use of internal positive control staining on each section (see Results) and the determination of ECD staining only on those areas used previously to grade the tumors after hematoxylin and eosin staining has led to a clearer correlation between lack of ECD expression and poor differentiation than described previously in other tumors.[13,30] Whereas we do not suggest that ECD expression is a pure differentiation marker, we are confident of the association described herein. Clearly, the follow up of patients with ECD-negative Dukes stage A and B tumors will be of interest. Irrespective of these results this is the first demonstration of the inverse association of ECD expression with progression and metastasis of colorectal carcinomas. The results also are in general agreement with demonstrations of ECD activity in other carcinomas.[11–14]

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