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Original Paper

Immunohistological Analysis of E-Cadherin, α-, β- and γ-Catenin Expression in Colorectal Cancer: Implications for Cell Adhesion and Signaling

B.M. Ghadimi,[1,3] J. Behrens,[2] I. Hoffmann,[4] W. Haensch,[3] W. Birchmeier[2] and P.M. Schlag[1]

[1]Department of Surgery and Surgical Oncology, Charité, Robert Rössle Hospital, Lindenberger Weg 80, 13122 Berlin, Humboldt University; [2]Max-Delbrück-Center of Molecular Medicine, Robert-Rössle-Str. 10, Berlin; [3]Section of Pathology, Robert Rössle Hospital, Humboldt University, Berlin; and [4]Unit of Statistical Analysis, Department of Surgery and Surgical Oncology, Charité, Robert Rössle Hospital, Humboldt University, Berlin, Germany

Intercellular adhesion mediated by the E-cadherin/catenin complex is a prerequisite for epithelial integrity and differentiation. In carcinomas, E-cadherin function is frequently disturbed, and has been suggested to increase invasion and metastasis of tumour cells. β-catenin has also been implicated in signaling pathways essential for tumour formation. We analysed the E-cadherin/catenin adhesion system of colorectal tumours at different clinical stages. In primary carcinomas ($n = 91$), there was a frequent reduction in E-cadherin (44%) and α-catenin expression (36%). In contrast, β-catenin and γ-catenin expression were seldom reduced (4% and 15%, respectively). Similar expression patterns were observed in liver metastases from unrelated colorectal tumours ($n = 27$). There was a significant relationship between loss of E-cadherin and α-catenin expression and poorly differentiated (G3–4) tumours. Our results suggest that reduction of E-cadherin/α-catenin expression is a frequent event in primary and metastatic colorectal carcinomas. Furthermore, β-catenin expression remains normal in colorectal cancer, suggesting the essential role of β-catenin in signaling pathways.

Key words: E-cadherin, catenins, colorectal cancer, metastasis
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INTRODUCTION

THE PROGNOSIS of colorectal cancer patients is determined by the development of metastases. The metastatic cascade starts with a breakdown of the epithelial integrity which enables tumour cells to leave epithelial structures, to invade the surrounding stroma, to enter either blood or lymphatic vessels and extravasate in the appropriate target organs.

Epithelial differentiation is critically dependent on the proper formation of intercellular junctions by cell–cell adhesion molecules. Impairment of the junctions allows invasion of epithelial cells and the progression of carcinomas [1]. Among the cell–cell adhesion molecules, E-cadherin is located at the zonula adherens and is a functional necessity for the integrity of epithelia [2, 3]. Cadherins are calcium-dependent transmembrane adhesion molecules which connect cells homotypically. *In vitro* studies in tumour cell lines have shown that disturbance of E-cadherin function is correlated with the acquisition of invasive properties [4–6]. Furthermore, decreased levels of E-cadherin expression have been noted in many immunohistochemical studies of epithelial cancers [7–12]. In some tumour types, including colorectal cancer, the loss of E-cadherin expression has been associated with loss of differentiated features in the tumour and has been found to correlate with an increased likelihood of distant metastases, suggesting a potential role for E-cadherin as an invasion or metastasis suppressor [8, 13]. Additionally, mutations of the E-cadherin gene have been described in gastric and breast cancer. These mutations lead

Correspondence to P.M. Schlag; e-mail: schlag@rrk-berlin.de
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to exon skipping with a diminished function of the calcium-binding regions [14] or to truncated fragments which are expected to be secreted [15]. In both cases the cell-adhesion activity of E-cadherin will be impaired.

The cytoplasmic domain of E-cadherin interacts with intracellular proteins called α-, β- and γ-catenins, which make contact with the microfilament network [2, 3]. The interaction of these molecules is the prerequisite for the proper formation of functionally intact adherens junctions. α-Catenin shows sequence similarity to vinculin and interacts with the cytoskeleton [16–18]. β-Catenin is the vertebrate homologue of the segment polarity gene armadillo of *Drosophila* and makes direct contact with the cytoplasmic domain of E-cadherin [19, 20]. γ-Catenin (plakoglobin) is closely related to β-catenin and is also found in desmosomal junctions [21–23]. Catenins are essential for E-cadherin function, and alterations in expression or structure of the catenins may lead to the disassembly of adherens junctions and the generation of more invasive cells. In some human cancers, such as that of the breast, oesophagus and prostate, decreased expression of α-catenin has been noted [24–26]. In some tumours, genetic alterations including homozygous deletions or localised mutations of the α-catenin gene account for the decrease of α-catenin expression. In addition, alterations in β- and γ-catenin expression and phosphorylation have been described for some tumour cell lines [27–29].

Besides their function in the adherens junctions, β-catenin and plakoglobin form complexes with the tumour-suppressor protein APC which are independent from the cadherin/catenin complex [30–32]. The *APC* gene is often mutated in colorectal cancer tissue. In familial adenomatosis coli (FAP) patients, *APC* is also altered as a germline mutation. In cells containing a mutated *APC*, intracellular β-catenin levels are elevated and can be reduced by reintroduction of wildtype *APC* [33]. Recently, it has been shown that β-catenin is frequently upregulated in adenomas and carcinomas of FAP patients, and a proportion of the protein is found in the cell nucleus [34].

β-Catenin has recently been shown to function as a transcription activator when complexed with members of the Tcf/LEF family of transcription factors [35, 36]. Additionally, it has been demonstrated that β-catenin forms permanent complexes with Tcf/LEF factors in colorectal cancer cell lines. Upregulation of β-catenin can occur as a result of mutations of *APC* (as indicated above) or mutations of β-catenin which prevent its downregulation [37, 38].

In vitro and *in vivo* data thus suggest a pivotal role of cadherins and catenins in various aspects of tumour progression. In this study we analysed by immunohistochemistry the expression of E-cadherin and α-, β- and γ-catenins in different clinical stages of colorectal tumours.

PATIENTS AND METHODS

Snap-frozen colorectal carcinoma tissue from patients who had undergone colectomy or liver resection in our department was used in this study. The resected metastases were not related to the primary tumours in this study. A portion of the tissue was used for routine histopathological examination by the pathologist. Serial sections of 5 μm were cut in a Cryocut 300 microtome (Leica Instruments, Nussloch, Germany), and immunohistochemically stained with the anti-E-cadherin monoclonal antibody 6F9 [5] and polyclonal antibodies against α-, β- and γ-catenin [39]. In addition, a monoclonal antibody (MAb) against β-catenin (Transduction Laboratories, U.K.) was used. Sections were stained using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique. Briefly, cryostat sections were air-dried for at least 2 h, fixed in acetone for 10 min at room temperature and preincubated with AB-serum for 20 min to block unspecific binding. The primary antibodies in appropriate dilution were applied for 60 min at room temperature. After each incubation, repeated washings (6 min) in tris-phosphate buffered saline (TBS) were performed. Subsequently, either rabbit-anti-mouse-IgG or mouse-anti-rabbit-IgG (DAKO) was applied for 30 min followed by the APAAP complex for another 30 minutes. The reaction was developed using naphthol AS-BI phosphate-fast red-levamisole diluted in tris buffer and slides were counterstained with Mayer's haematoxylin for 30–45 sec and mounted with Kaiser's glycerol gelatin.

The expression of E-cadherin and the catenins was evaluated without knowledge of the clinical and histopathological parameters by two independent observers and scored as 'normal' when more than 80% of the epithelial cells showed linear intercellular staining, and 'reduced' when less than 80% of the cells expressed intercellular staining. In every case, normal mucosa from the same patient was stained as a control.

Histological findings and statistics

To correlate the expression of E-cadherin, α-, β- and γ-catenin with conventional histopathological parameters, the tumour stage was determined according to the UICC classification (International Union against Cancer). The following parameters were compared: depth of invasion (pT), lymph node involvement (pN), grade of tumour differentiation (G) and blood and lymphatic vessel invasion (V and L). For statistical analysis the Pearson Chi-square test was used.

RESULTS

Expression of E-cadherin, α-, β- and γ-catenin in primary colorectal carcinomas

Expression of E-cadherin, α-, β- and γ-catenin was clearly evident at the cell-cell boundaries of epithelial cells in normal colorectal mucosa (Figure 1). Mesenchymal tissue surrounding the epithelial cells did not express E-cadherin or any of the catenins. In the colorectal carcinomas ($n = 91$), various staining patterns were observed; i.e. tumours with normal (Figure 2a), heterogenous (Figure 2b) and absent (Figure 2c) expression were found. E-cadherin and all three catenins were expressed in a similar manner to normal mucosa in 33 of 91 carcinomas (36%, Figure 3a–d). With four exceptions, these tumours were well to moderately differentiated. In the other 58 carcinomas, the expression of at least one of the investigated molecules was impaired. A reduction of E-cadherin and α-catenin expression was seen in 44% (40/91) and 36% (33/91) of all investigated tumours, respectively (Figure 3e–f, Table 1). Interestingly, a simultaneous reduction of E-cadherin and α-catenin was observed in 21% (19/91) of these cases (Table 1). Remarkably, in six of the ninety-one tumours, epithelial structures existed despite a lack of E-cadherin expression (Figure 3e). In contrast to the marked reduction in E-cadherin and α-catenin, γ-catenin was found to be reduced in only 15% (14/91) of cases. The expression of β-catenin was even more stable, being reduced only in 4% (4/91) of the cases (Figure 3g, h). This stable expression of β-catenin was confirmed by staining with an additional anti-β-catenin antibody (data not shown).

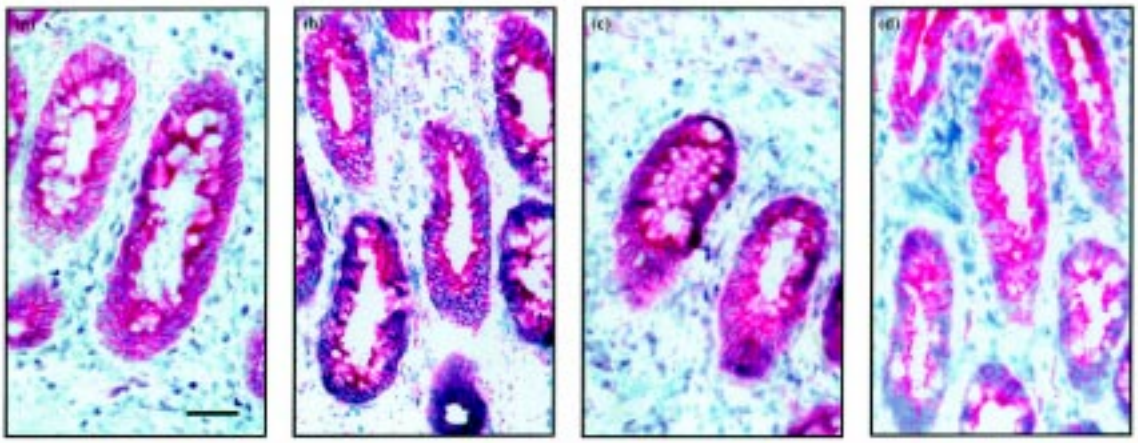

Figure 1. Expression of E-cadherin and the catenins in normal colorectal mucosa. Immunostained sections for E-cadherin (a), α-catenin (b), β-catenin (c) and γ-catenin (plakoglobin) (d). Expression of all four components was confined to the cell borders of epithelial cells, with no expression in the surrounding mesenchymal cells. Bar represents 75 μm.

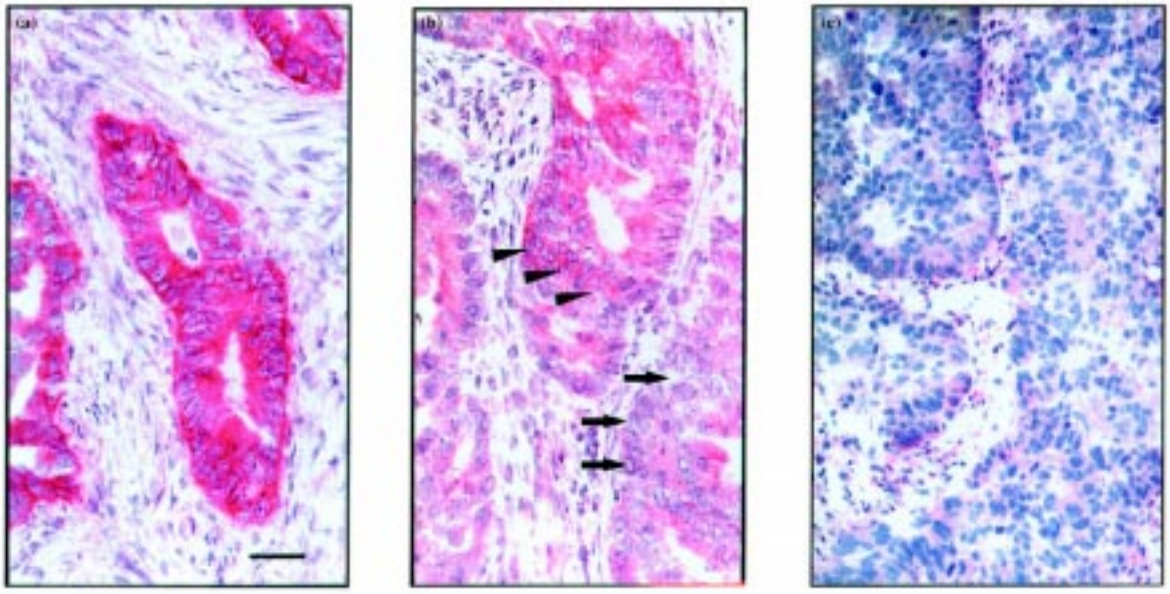

Figure 2. Variation of E-cadherin expression in different colorectal tumours. Representative examples of tumors with normal (a), reduced (b) and absent expression (c) of E-cadherin are shown. Note that in (b) E-cadherin expression was heterogenous, being focally preserved in certain areas of the tumors (arrowheads) and lacking in others (arrows). (a) and (b) were moderately differentiated (G2) whilst (c) was a poorly differentiated (G3) with absent expression of E-cadherin. Bar represents 50 μm.

Expression of E-cadherin, α-, β- and γ-catenin in colorectal cancer liver metastases

We investigated the expression of E-cadherin and the catenins in 27 liver metastases derived from colorectal cancers different to the afore mentioned primary tumours. E-cadherin expression was reduced in 48% (13/27) and α-catenin in 36% (10/27) of the cases. As in primary colorectal carcinomas, the expression of β- and γ-catenin was preserved in colorectal liver metastases in more than 85% (23/27) and 96% (26/27) of the cases, respectively.

Table 1. *Co-expression pattern of E-cadherin and α-catenin in colorectal carcinomas* (n = 91)

E-Cadherin	α = Catenin	(*n*)
Normal	Normal	41% (37)
Reduced	Normal	23% (21)
Reduced	Reduced	21% (19)
Normal	Reduced	15% (14)

Correlation of expression with histopathological parameters

Correlating these data with respect to depth of invasion (pT), there was no significant correlation between the loss of E-cadherin expression and depth of invasion (Table 2). However, 4 of the 5 pT1 carcinomas (80%) had normal E-cadherin expression, compare with 7 of the 13 pT4 tumours (54%). No significant association between expression of any catenin and depth of invasion was seen (Table 2). A significant correlation was found between tumour grade and loss of E-cadherin ($P = 0.03$) or α-catenin ($P = 0.008$) expression (Table 2). More than 60% of well- to moderately-

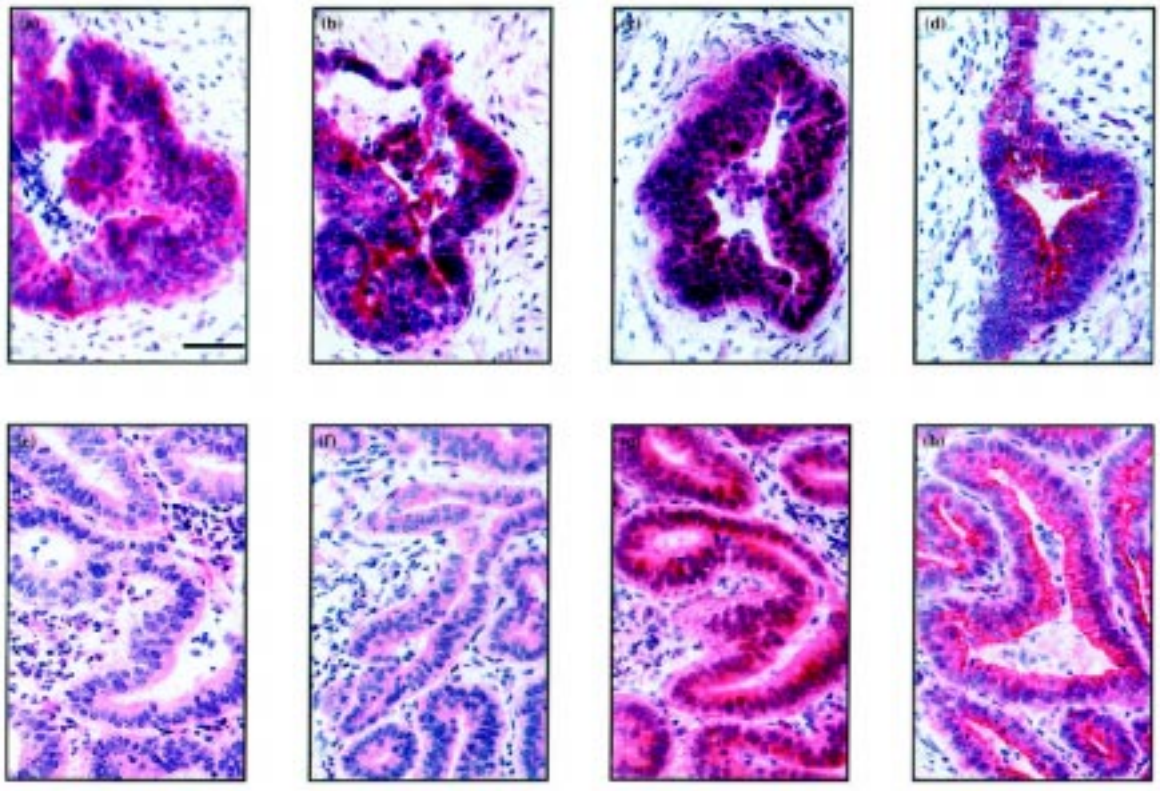

Figure 3. Expression of E-cadherin and the catenins in colorectal tumours. Comparison of two colorectal carcinomas exhibiting different expression patterns of E-cadherin (a, e), α-catenin (b, f), β-catenin (c, g) and γ-catenin (d, h). In one tumour (a–d) expression of all four components was preserved, whereas in the other (e–f) E-cadherin and α-catenin were lacking whilst β-catenin and γ-catenin (g, h) were preserved. Note that although this tumour had preserved epithelial structures, E-cadherin expression was absent. Bar represents 75 μm.

differentiated carcinomas (G1-2) had normal E-cadherin and α-catenin expression compared with only 31% of poorly differentiated tumours (G3-4). In contrast, β- and γ-catenin expression was normal in the vast majority of the cases, with no significant correlation between reduced expression and tumour grade.

DISCUSSION

In the present study, we examined the expression of E-cadherin and its intracellular binding partners α-, β- and γ-catenins in primary colorectal carcinomas, and found that expression of E-cadherin and α-catenin was frequently reduced, whereas β- and γ-catenin expression remained

Table 2. Correlation of E-cadherin and α-catenin with depth of invasion (pT category) and degree of differentiation (grade) of colorectal carcinomas

	Depth of invasion (pT-category)				Differentiation (grade)		
	T1 *n* (%)	T2 *n* (%)	T3 *n* (%)	T4 *n* (%)	G1-2 *n* (%)	G3-4 *n* (%)	*P* value*
E cadherin							
Normal	4 (80)	16 (67)	25 (51)	7 (54)	49 (63)	4 (31)	
Reduced	1 (20)	8 (33)	24 (49)	6 (46)	29 (37)	9 (69)	0.03
α-catenin							
Normal	4 (80)	15 (63)	30 (61)	9 (69)	54 (69)	4 (31)	
Reduced	1 (10)	9 (38)	19 (39)	4 (31)	24 (31)	9 (69)	0.008
β-catenin							
Normal	5 (100)	23 (96)	48 (98)	12 (92)	76 (97)	12 (92)	
Reduced	0	1 (4)	1 (2)	1 (8)	2 (3)	1 (8)	ns
γ-catenin							
Normal	5 (100)	17 (71)	41 (84)	13 (100)	67 (86)	9 (69)	
Reduced	0	7 (29)	8 (16)	0	11 (14)	4 (31)	ns

There was no significant reduction in expression of E-cadherin or any of the catenins with increased depth of invasion (*P* values not shown). **P* value for G3-4 compared with G1-2; Pearson. Chi-square test; ns, non-significant.

normal. Similar results were obtained in colorectal liver metastases. There was a significant relationship between reduced E-cadherin/α-catenin and lower tumour grade.

Various studies have shown that tumour invasion and formation of metastases is associated with an impairment of expression or structure of the E-cadherin/catenin adhesion complex [1–3]. Further, functional data demonstrate that E-cadherin controls invasiveness *in vitro* and metastasis formation *in vivo* [4–6, 40]. We found that E-cadherin was down-regulated in approximately 44% of colorectal cancers. A similar loss of E-cadherin expression has been reported in other epithelial tumours such as breast, gastric, head and neck and prostate carcinomas [7, 9, 12, 41]. We also investigated the expression of the catenins which are essential for E-cadherin function [24–26], and showed that only α-catenin expression was significantly reduced in colorectal tumours, whilst β- and γ-catenins expression was generally normal. These results concur with results of a previous study where a greater reduction of α-catenin was described [42]. α-Catenin is important for the linkage of the cadherins to the cytoskeleton. The loss of expression of α-catenin in the tumours is likely to disturb cell–cell adhesion in a manner similar to loss of E-cadherin.

A clear correlation between loss of E-cadherin/α-catenin expression and depth of tumour infiltration into the intestinal wall (T-category) was not found, possibly owing to the small numbers, especially in the pT1 group. In contrast, we were able to demonstrate a significant correlation between the histopathological grading of the tumours and increased loss of E-cadherin and α-catenin expression. Defective expression was significantly more frequent in less differentiated carcinomas (G3-4) with pronounced loss of epithelial morphology than in the better differentiated tumours (G1/G2). Grade of differentiation is an important prognostic factor in colorectal cancer influencing patients' survival.

For a considerable proportion of tumours the expression of the cadherin/catenin complex was apparently not disturbed. In these cases other mechanisms supporting invasiveness may play a role, e.g. expression of motility factors, such as scatter factor [43] and/or mutational inactivation of the adhesion components. Mutations of the E-cadherin gene have been described in gastric and breast carcinomas, which lead to proteins with diminished function [14, 15]. Some of the tumors in our study lacked E-cadherin expression but had preserved epithelial structures. In such cases, other cadherins expressed in the intestinal tract, such as OB-, P- or R-cadherins [11, 44–46], may functionally compensate. Whether colorectal carcinomas can be classified according to their preservation of the cadherin/catenin complex remains to be determined.

In contrast to the frequent reduction of E-cadherin and α-catenin expression, γ- and to an even greater extent β-catenin expression remained normal in colorectal primary carcinomas and liver metastases. In particular, β-catenin expression was normal independently of tumour infiltration or differentiation. This result is in striking contrast to a recently published series which demonstrated a significant reduction of β-catenin in colorectal cancer [47]. Whether this difference is due to the different β-catenin antibody or detection system used remains to be determined. Because the staining observed with the APAAP method is stronger, slight reductions in β-catenin expression might not be so noticeable. However, β-catenin is not only involved in cell–cell adhesion but also in the wnt/wingless pathway and is negatively regulated by the APC protein. Mutations of APC, which are observed in the majority of colorectal cancers, result in stabilisation of β-catenin, whilst β-catenin mutations can also contribute to high β-catenin levels [37]. As summarised in a recent review by Hirohashi [48], β-catenin might be increased and/or stabilised in human cancers, thereby stimulating wnt/wingless pathways. According to our results, we presume that elevated β-catenin levels might be important in signaling pathways in neoplastic cells. The interaction between β-catenin mediated cell–cell adhesion and signaling pathways needs further investigation.

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