Biochemical Analysis and Subcellular Distribution of E-Cadherin-Catenin in Adenocarcinomas of the Gastro-Oesophageal Junction

B.P.L. WIJNHOVEN1, E.T. TUCKER2, W.N.M. DINJENS3, H.W. TILANUS1 and M. PIGNATELLI2

Departments of 1Surgery and 3Pathology, Erasmus MC, Rotterdam, The Netherlands; 2Division of Histopathology, Department of Pathology & Microbiology, University of Bristol, Bristol Royal Infirmary, Bristol, United Kingdom

Abstract. Background: Disturbances in the expression or structure of E-cadherin-catenin, a cell-cell adhesion complex, perturb its cell adhesive function. Materials and Methods: We studied the expression and distribution of the E-cadherin-catenin complex in 24 adenocarcinomas of the gastro-oesophageal junction (GOJ) by immunohistochemistry and Western blotting of the Triton X-100-soluble (membrane bound) and insoluble fractions (cytoskeleton bound). Results: Immunohistochemistry demonstrated redistribution of E-cadherin, α-, β- and γ-catenin from the membrane to the cytoplasm in 13/24 (54%), 18/24 (75%), 16/24 (67%) and 15/24 (63%) tumours, respectively. Five tumours showed nuclear localisation of β-catenin. Western blotting showed redistribution between the TX-100 soluble and insoluble fraction of E-cadherin and the catenins in 5/11 (45%), 4/10 (40%), 5/11 (45%) and 5/11 (45%) tumours, respectively. Conclusion: Loss of membrane bound E-cadherin-catenin is frequently observed in adenocarcinomas of the GOJ and this may reflect loss of function of the E-cadherin-catenin complex in these cancers.

The cadherins are a family of calcium-dependent transmembrane adhesion proteins that interact in a homotypic fashion, playing a central role in the maintenance of tissue integrity, tissue morphology and cell-cell recognition (1, 2). The importance of E( epithelial)-cadherin expression and function in normal development and tissue function is demonstrated by the lethality of E-cadherin knockout mice at an early stage in embryogenesis (3). E-cadherin is bound via a series of undercoat proteins, the catenins, to the actin cytoskeleton. The catenin family comprises α-, β- and γ-catenin and a recently discovered 120 KDa protein, p120catenin (p120ctn). β- or γ-catenin bind directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner. α-catenin then links β- or γ-catenin to the actin microfilament network of the cytoskeleton. The linkage between transmembranous E-cadherin, the catenins and the actin filaments is necessary to form strong cell-cell adhesion. Deletion of the intracellular catenin-binding domain of E-cadherin or alterations in the functionally active catenins, results in loss of the ability of E-cadherin to establish cell-cell adhesion (4-7).

Several studies have implicated E-cadherin in both the early and late stages of tumour initiation and progression. Various human cancer cell lines with an epithelial, differentiated morphology were generally non-invasive and expressed E-cadherin, whereas cell lines with a fibroblast-like morphology were invasive and had often lost E-cadherin expression (8, 9). Using a transgenic mouse model of pancreatic β-cell tumourigenesis, Perl et al. demonstrated that loss of E-cadherin mediated cell-cell adhesion is causally involved in the transition from adenoma to carcinoma (10). Immunohistochemical studies in human cancers have frequently shown that a proportion of invasive carcinomas and carcinomas in situ show aberrant levels of E-cadherin and catenin expression in comparison to their related normal tissue (11-13). Therefore, changes in E-cadherin-catenin expression appear to be an important step in the development and progression of a malignant tumour.

Adenocarcinomas of the distal oesophagus and gastric cardia (i.e. gastro-oesophageal junction(GOJ)) are thought to arise from (short) segments of premalignant, metaplastic columnar epithelium, also referred to as Barrett’s oesophagus. Most carcinomas carry a poor prognosis as local and systemic invasion is often seen at early stages. This is worrisome, since adenocarcinomas of the GOJ show an
increase in incidence throughout the Western world, especially among middle-aged white males, surpassing that of any other malignant tumour (14-17).

To elucidate the role of the E-cadherin-catenin complex in adenocarcinomas of the GOJ, we evaluated the level of expression and distribution of E-cadherin and the catenins in tumour tissue versus the normal mucosa of the same patients. E-cadherin and catenin are known to be present in the cell in different protein pools, membrane bound, free cytoplasmic and cytoskeleton bound. The expression of E-cadherin and catenins and their biochemical distribution between the Triton-X-100 soluble (membrane bound) fraction and the Triton-X 100 insoluble (cytoskeleton bound) fraction was assessed using fractional protein extraction and Western blot analysis. The spatial distribution of the proteins between the cell membrane and cytoplasm and nucleus was assessed using immunohistochemistry.

Materials and Methods

Patients and samples. Twenty-four patients with adenocarcinoma of the GOJ (i.e. distal oesophagus or gastric cardia), treated at the Erasmus MC, Rotterdam, The Netherlands, were included in our study. There were 20 males and 4 females with a median age of 63 years (range 43-78 years) at the time of operation. All patients underwent transthoracic resection of the oesophagus and proximal stomach and the gastro-intestinal tract was reconstructed with a gastric tube in all cases, as described previously (18). None of the patients received (neo) adjuvant therapy.

After routine pathological examination of the resection specimens, primary tumour samples and corresponding normal tissue was stored as archival paraffin blocks. From 11 patients also small pieces of the tumour and surrounding normal oesophageal or gastric mucosa were available as fresh frozen tissue. Fresh tissue was obtained immediately postoperatively and stored in liquid nitrogen at the department of Pathology, Erasmus MC.

The group of 24 tumours consisted of one pT1 tumour, 4 pT2 tumours and 19 pT3 tumours. Six tumours were lymph node-negative (pN0) and 18 tumours had positive lymph nodes (pN1). Eight tumours had a good to moderate grade of differentiation and 16 tumours had a poor tumour grade. In 10 tumours signet ring cells could be detected. Classification of the tumours according to the pTNM criteria for carcinoma of the oesophagus established by the International Union Against Cancer (UICC 1997) revealed one tumour of stage I, 6 tumours stage II (IIA and IIB), 6 tumours stage III and 11 tumours of stage IV.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue blocks of the primary tumour samples were cut into 4-mm thick sections, mounted on AAS-coated glass slides and stained using a standard avidin biotin immunoperoxidase technique. Sections were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase was blocked by incubating the sections in 0.5% hydrogen peroxide in methanol for 20 minutes. To enhance immunoreactivity, dehydrated sections were treated with an antigen retrieval solution in a microwave oven. The slides were submerged in 0.01 M citrate buffer at pH 6.0 and were heated in a 700W microwave on full power for 15 minutes.Slides were then pre-incubated with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS/BSA 5% prior to incubation of the primary antibody at 4°C overnight. The primary antibodies used were E-cadherin (Euro-Diagnostica, Arnhem, The Netherlands) and α-catenin, β-catenin and γ-catenin (Transduction Laboratories, Lexington, USA). Subsequently, the slides were washed in PBS and incubated with biotinylated goat anti-mouse IgG (Biogenex, San Ramon, USA) for 30 minutes. After three washes in PBS, the slides were incubated with streptavidin-peroxidase complex (Biogenex) for a further 30 minutes and finally developed with activated 3,3'-diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.08% H2O2 solution for 7 minutes. The slides were then counterstained with Mayer’s Haematoxylin and dehydrated in alcohol before mounting. Negative control sections were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins. Positive controls using normal gastric epithelium were also run with each batch, in addition to using non-involved normal oesophageal or gastric mucosa as an internal positive control.

Western blotting. Expression of E-cadherin-catenin was analysed by Western blotting in 11 primary tumours and corresponding normal mucosa. Due to insufficient frozen tissue, α-catenin expression could not be assessed in a single tumour. Sections of 5-μm were cut on the cryostat and stained with H&E to be compared with the formalin-fixed paraffin-embedded tissue sections. The frozen tissue samples were dissected to exclude as much stromal tissue as possible and trimmed in order to get the same dimensions, to ensure that an approximately similar amount of tissue was being analysed from each specimen. Approximately twenty-sections of 15-mm thickness were then cut from each sample and placed in a pre-chilled eppendorf.

Tissue sections were solubilised by cytoskeleton buffer (19). Three hundred μl of soluble fraction lysis buffer (0.5% Triton X-100, 50 mM sodium chloride, 10 mM Pipes, 3 mM magnesium chloride, 300 mM sucrose, protease inhibitor tablets (1 tablet/50 mL lysis buffer; Boehringer Mannheim, Mannheim, Germany) were added to each tube. The tubes were kept on ice for 20 minutes with repeated vortexing, then were centrifuged at 13,000 rpm for 10 minutes at 4°C. The resulting supernatant (with the Triton X-100 soluble fraction of the cell protein) was collected. The pellet was lysed in 200μl of the sodium dodecyl sulphate (SDS) lysis buffer (15 mM Tris-HCl, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) at 100°C for 15 minutes and then spun at 13,000 rpm for 10 minutes at 4°C. The supernatant (with the Triton X-100 insoluble cell protein fraction) was collected. Samples were either analysed immediately or frozen at -70°C.

The protein concentration was measured using the Bradford protein assay kit (Biorad, CA, USA). Equal protein loading of corresponding lanes ensured accurate comparison of protein expression of the tumour and normal tissues by comparing band sizes and intensities. The calculated volumes of lysates were denatured and reduced with sample buffer (2% SDS, 10% glycerol, 50mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 0.25% bromophenol blue), boiled for 10 minutes and then resolved by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 8% polyacrylamide gel. The resolved proteins were transferred by electroblotting onto nitro-cellulose membranes.
(Millipore, Herts, UK). The membranes were blocked with 5% non-fat dried milk (Marvel) in Tris-buffered saline-Tween 20, pH 7.2 for 1 hour at room temperature and then incubated with the primary antibody diluted in TBS-T overnight at 4°C. Antibodies were used at a dilution of 1μg/mL for E-cadherin, β- and γ-catenin and 4μg/mL for α-catenin. The blot was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Dako, Glostrup, Denmark) at 1μg/mL for 2 hours at room temperature. Detection was by enhanced chemiluminescence reagent (ECL, Amersham Life Sciences, Bucks, UK) followed by autoradiography with Hyperfilm-MP (Amersham Life Sciences).

**Evaluation of immunostaining and Western blot.** The stained sections were examined by two observers under light microscopy. The localisation and intensity of staining was always assessed relative to the normal mucosa in the same section. Tumours displaying well localised membranous staining and weak cytoplasmic staining were considered as normal. Weak or absent membranous staining, intense cytoplasmic and nuclear staining were considered abnormal (20). In case of tumour heterogeneity, those areas within the tumour that showed the lowest membrane staining and/or highest cytoplasmic staining were assessed and analysed further.

The expression of the E-cadherin-catenin complex in tumour tissue and corresponding normal tissue was analysed for the soluble and insoluble fraction. The ratio of the signal-derived intensity of tumour and normal tissue was determined and compared between the soluble and insoluble fraction. Western blots were classified as abnormal when there was a difference in ratio between the soluble and insoluble fraction.

**Statistical analysis.** Categorical data were displayed in contingency tables when analysing protein expression with respect to pathological data, and data obtained from Western blotting, and analysed by Chi-square or Fisher’s exact test where appropriate. A p-value of <0.05 was accepted as statistically significant.

**Results**

**Immunohistochemistry: expression and localisation of E-cadherin-catenin.** Normal squamous epithelium of the oesophagus and normal gastric epithelium showed a strong cadherin-catenin in 54-75% of adenocarcinomas are similar to other studies (22-24). Reduced membranous localisation of E-cadherin and the catenins is associated with increased invasiveness, lymphatic involvement and prognosis (23). However, we did not find a correlation between abnormal expression of E-cadherin and the catenins, as determined by immunohistochemistry, already occur in dysplastic Barrett’s epithelium and are considered to be early events in carcinogenesis (22). Our findings of reduced membranous expression and/or increased cytoplasmic staining of E-cadherin-catenin in 54-75% of adenocarcinomas are similar between immunohistochemistry and Western blotting: both techniques detected 5/11 adenocarcinomas with a normal expression/distribution of E-cadherin. However, one tumour with a normal membranous expression of E-cadherin on immunohistochemistry, had an abnormal distribution of the protein between the soluble and insoluble fraction as shown by Western blotting, and vice versa (Figure 3A). There was no correlation between immunohistochemistry and Western blotting in detecting abnormal expression and localisation of the catenins (Figures 3B-D).

**Discussion**

Unlike colorectal carcinomas, there is no uniform genetic model that fits the histological progression from premalignant dysplastic Barrett’s epithelium towards invasive adenocarcinoma of the GOJ (21). However, it has become increasingly apparent that the E-cadherin-catenin cell-cell adhesion complex plays an important role in tumour initiation and progression. Changes in expression of E-cadherin and the catenins, as determined by immunohistochemistry, already occur in dysplastic Barrett’s epithelium and are considered to be early events in carcinogenesis (22). Our findings of reduced membranous expression and/or increased cytoplasmic staining of E-cadherin-catenin in 54-75% of adenocarcinomas are similar to other studies (22-24). Reduced membranous localisation of E-cadherin and the catenins is associated with increased invasiveness, lymphatic involvement and prognosis (23). However, we did not find a correlation between abnormal expression of E-cadherin-catenin and unfavourable tumour characteristics, probably due to the relatively small numbers of tumours analysed in our study.

A significant proportion of tumours demonstrated strong nuclear expression of β-catenin and one tumour with nuclear localisation of γ-catenin. Nuclear accumulation of β-catenin is an early event in the neoplastic progression of Barrett’s oesophagus since this is already observed in low-grade dysplastic Barrett’s epithelium (25, 26). Nuclear localisation of both catenins indicates their role in signal transduction and regulation of gene expression (27). Free, cytoplasmic β-catenin translocates to the nucleus, binds transcription factors (Tcf/LE) and stimulates transcription of target genes such as c-myc and cyclin D1(28, 29).
Figure 1. E-cadherin immunoreactivity in normal gastric epithelium (A); E-cadherin immunoreactivity in a tumour with membranous localisation of the protein (B); Reduced membranous expression of E-cadherin (C); Cytoplasmic staining for α-catenin (also note the immunoreactivity of a vessel) (D); Reduced membranous and increased cytoplasmic staining of β-catenin (E and F); Strong cytoplasmic and homogeneously nuclear expression of β-catenin (G); Scattered nuclear expression for γ-catenin (F). Original magnification X400.
In the present study we also evaluated the biochemical and spatial distribution of E-cadherin, α-, β- and γ-catenin in the different cell compartments in cancerous tissues and normal adjacent mucosa from the same patient. We show, in agreement with other studies, that E-cadherin, α-, β- and γ-catenin are present in two protein pools, a Triton X-100 soluble membrane bound fraction and a Triton X-100 insoluble cytoskeleton bound fraction (30, 31). Our results also demonstrate that complex disturbances of E-cadherin and catenins expression are common in adenocarcinomas of the GOJ. Evaluating the distribution of E-cadherin and catenins between the soluble and insoluble fraction showed an increase or decrease in expression in one of the two fractions relative to the normal mucosa. This shows that expression of the proteins does not always imply that they are functioning, as binding of the E-cadherin-catenin complex to the cytoskeleton is essential for its role in cell adhesion. Two studies demonstrated increased β-catenin in the soluble fraction and decreased β-catenin in the insoluble fraction in a subset of oesophageal squamous cell carcinomas and colorectal carcinomas and this was associated with increased cytoplasmic staining by immunohistochemistry (32, 33). We confirm their findings, since in 4/5 tumours with an abnormal Western blot for β-catenin, immunohistochemistry revealed reduced membranous and increased cytoplasmic staining. Moreover, 3/5 tumours had strong nuclear staining for β-catenin.

Failure of E-cadherin and the catenins to localise to the membrane and/or bind the cytoskeleton in spite of their abundance may be due to genetic or epigenetic changes in their structure and/or function. E-cadherin, α, β and γ-catenin mutations have been reported in different tumours and cell lines (5, 34-36). However, we and others could detect neither E-cadherin nor β-catenin gene mutations underlying the aberrant expression of the proteins (25, 37, 38). Post-translational regulation, perhaps by tyrosine phosphorylation, may be responsible for the distribution of the catenins in the different pools. The phosphorylation status of the catenins may be affected by intracellular membrane associated tyrosine kinases like src and receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor. In response to transfection with v-src, β-catenin is phosphorylated and phosphorylation may interfere with cadherin function and disrupt adherens junctions, without affecting the overall expression of either the catenins or E-cadherin (39, 40). Moreover, E-cadherin tyrosine de-phosphorylation was observed in primary bronchopulmonary and thyroid carcinomas displaying a spotty cytoplasmic pattern or a pericellular redistribution of the complex with no synthesis variations for E-cadherin or the catenins (41, 42).

Immunohistochemistry and Western blotting data correlated when expression and distribution of E-cadherin was analysed. E-cadherin is expressed in epithelia only. On the contrary, the
Catenins are also expressed in non-neoplastic cells, such as muscle, endothelium and nerves (Figure 1D). Therefore, contamination of the protein lysates with stromal tissue can influence the signal-derived intensity of the Western blots. This might explain why there was no correlation between the results of the immunohistochemistry and the Western blotting with respect to the catenins. On the other hand, all tumour tissue samples were carefully selected to contain at least 75% tumour cells with minimal stromal tissue. Secondly, Western blotting detected tumour samples with redistribution of the E-cadherin-catenin complex that was shown normal by immunohistochemistry. Therefore, both techniques might be of additional value in detecting discrete but important changes in distribution of the cell-cell adhesion complex in cancerous tissues.

In summary, our study showed that a perturbation in the expression, distribution and hence function of the members of the E-cadherin-catenin complex is frequently seen in adenocarcinomas of the gastro-oesophageal junction. Immunohistochemical technique and Western blotting of the soluble and insoluble fraction of the proteins are both valuable tools in detecting aberrant expression and distribution of the complex and even might have additional value. Further research will have to focus on the possible mechanisms underlying the redistribution of this cell-cell adhesion complex in cancers. Ultimately, this might improve our understanding of the aggressive features of adenocarcinomas of the gastro-oesophageal junction, which have shown a sharp increase in incidence over the past decades in the Western world.

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References

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