

## E-cadherin gene mutations are rare in adenocarcinomas of the oesophagus

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**Summary** Reduced expression of E-cadherin, a cell–cell adhesion molecule, is observed in oesophageal adenocarcinomas and correlates with less favourable pathological parameters and survival. To determine if genetic events lead to reduced E-cadherin expression in these patients, we screened all 16 exons of the E-cadherin gene for mutations with the polymerase chain reaction single-strand conformation polymorphism analysis (PCR-SSCP) technique in 49 resection specimens, including four loco-regional lymph node metastases, four established cell lines and four xenografts. Fifteen exon-spanning primer pairs were used, and in nine amplicons aberrant bands were detected. Sequencing of the amplicons revealed a one base-pair deletion (codon 120; exon 3) in cell lines JROECL 47 and JROECL 50 leading to a premature downstream stop codon. Polymorphisms were identified for amplicons 1, 4/5, 11, 12, 13, 14 and 16 corresponding with data from the literature. Three new polymorphisms were detected for amplicons 2, 3 and 4/5. Loss of heterozygosity (LOH) of the E-cadherin locus on 16q22.1 was examined with four polymorphic markers. LOH was found in 31 of the 48 informative cases (65%). These results show that, despite the frequent LOH of the E-cadherin locus, mutations in the E-cadherin gene are rare events and can not be held responsible for down-regulation of E-cadherin observed in the majority of adenocarcinomas of the oesophagus.

**Keywords:** adenocarcinoma oesophagus; E-cadherin; mutations; PCR-SSCP; LOH

Cadherins are a family of  $\text{Ca}^{2+}$ -dependent cell–cell adhesion molecules. E-cadherin is a transmembrane protein with five tandemly repeated extracellular domains and a cytoplasmic domain that connects to the actin cytoskeleton through a complex with  $\alpha$ -,  $\beta$ - and  $\gamma$ -catenins. E-cadherin is expressed on the cell surface in most epithelial tissues and is important for establishing cell polarity, maintaining epithelial integrity and cellular differentiation (Takeichi, 1991). The potential for E-cadherin to serve as an invasion or metastatic suppressor in epithelial tumorigenesis has been elucidated from in vitro studies. E-cadherin-negative epithelial cells grow invasive with a mesenchymal phenotype. After transfection with E-cadherin cDNA, epithelial structure is restored (Frixen et al, 1991; Vleminckx et al, 1991).

Consistent with this observation is a reduced or absent E-cadherin expression in various epithelial cancers showing invasive growth (Gabbert et al, 1996; Kuczyk et al, 1998; Sulzer et al, 1998). Decreased expression of E-cadherin in Barrett's oesophagus, adenocarcinomas of the oesophagus and gastro-oesophageal junction was found to be related with progression of Barrett's oesophagus to adenocarcinoma, tumour stage and lymph node metastasis (Bongiorno et al, 1995; Bailey et al, 1998). Furthermore, E-cadherin expression was an independent variable predicting survival in patients after resection for adenocarcinomas of the oesophagus (Krishnadath et al, 1997).

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Loss of E-cadherin expression resulted in the transition from well-differentiated adenoma to invasive carcinoma in mouse pancreatic  $\beta$ -cell carcinogenesis (Perl et al, 1998). Inactivating mutations in the E-cadherin gene have been described for various tumours such as lobular breast cancer (*in situ*) and diffuse infiltrating gastric cancer concomitant with loss of heterozygosity (LOH) at the E-cadherin locus in E-cadherin-negative tumours (Becker and Hofler, 1995; Berx et al, 1995, 1996; Vos et al, 1997). Moreover, two recent studies showed that germline mutations in the E-cadherin gene are associated with early onset familial gastric cancer (Gayther et al, 1998; Guilford et al, 1998). Whether genetic alterations in the E-cadherin gene play a role in the pathogenesis of adenocarcinomas of the oesophagus and gastro-oesophageal junction is not known. Therefore, we screened adenocarcinomas of the oesophagus and gastro-oesophageal junction for E-cadherin gene mutations and LOH.

## METHODS

### Tumour specimens

Fresh samples of adenocarcinomas of the distal oesophagus or gastro-oesophageal junction were obtained from 45 resection specimens. For analysis the tumour samples were microdissected to enrich for cancer cells (> 75%). Nineteen tumours showed microscopic evidence of surrounding intestinal metaplasia indicative for Barrett's carcinomas. Samples of tumour and normal gastric epithelium or squamous epithelium of the oesophagus were snap-frozen and stored in liquid nitrogen. Four lymph nodes infiltrated by tumour were frozen as well.

## Cell lines and xenografts

In vitro cell lines from three adenocarcinomas JROECL19, JROECL33, JROECL50 and one adenosquamous carcinoma JROECL47 established by Rockett et al (1997) were obtained from European Collection of Cell Cultures (ECACC). In vivo xenografts were obtained after transplantation of tumour tissue to female nude mice, 4–6 weeks of age. From three lymph node metastases M2.1X1, M9X1, M4.1X2 and one primary tumour P23X1, xenografts were obtained.

## DNA preparation

DNA from cell lines was isolated according to standard procedures. Genomic DNA from xenografts and tumour samples and normal tissue was isolated from consecutive 5-μm cryostat tissue sections by overnight proteinase K incubation at 55°C followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE (10 mM Tris-HCl, pH 7.8; 1 mM EDTA). The tumour tissue samples contained at least 75% tumour cells.

## Analysis of the E-cadherin gene by PCR-SSCP

The entire open reading frame of the E-cadherin gene was screened for mutations using 15 exon-spanning primer pairs (Berk et al, 1995). Genomic DNA was used at 50–100 ng per 15 μl reaction mixture containing 1.5 mM magnesium chloride, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8 μCi α-<sup>32</sup>PdATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer and 0.2 U *Taq* polymerase (Promega, Madison, WI, USA). Each PCR was overlaid with mineral oil. PCR was performed for 35 cycles (denaturing at 95°C for 30 s, annealing at the appropriate temperature for 45 s and extension at 72°C for 1 min). A final extension step was carried out at 72°C for 10 min. PCR products were diluted 1:4 with a loading buffer (95% formamide, 10 mM EDTA (pH 8.0), 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95°C for 4 min and snap-cooled on ice. Appropriate aliquots of the radiolabelled PCR products were separated on a non-denaturing polyacrylamide gel (6% polyacrylamide) containing 10% glycerol and run at 7 W overnight at room temperature in 1 × TBE running buffer. Gels were fixed in acetic acid (10%), dried on blotting paper (Schleicher & Schuell, Dassel, Germany) on a vacuum gel dryer and exposed to X-ray film overnight at -70°C, using intensifying screens. DNA with aberrantly migrating PCR-SSCP fragments was reamplified and purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into a PGEM-T easy vector (Promega, Madison, WI, USA), and sequenced with α-<sup>35</sup>SdATP according to the dideoxy chain termination method. Electrophoresis of the sequenced samples was carried out on an 8% denaturing polyacrylamide gel. After fixation and drying, gels were exposed to X-ray film for 1–3 days at room temperature.

## LOH determination

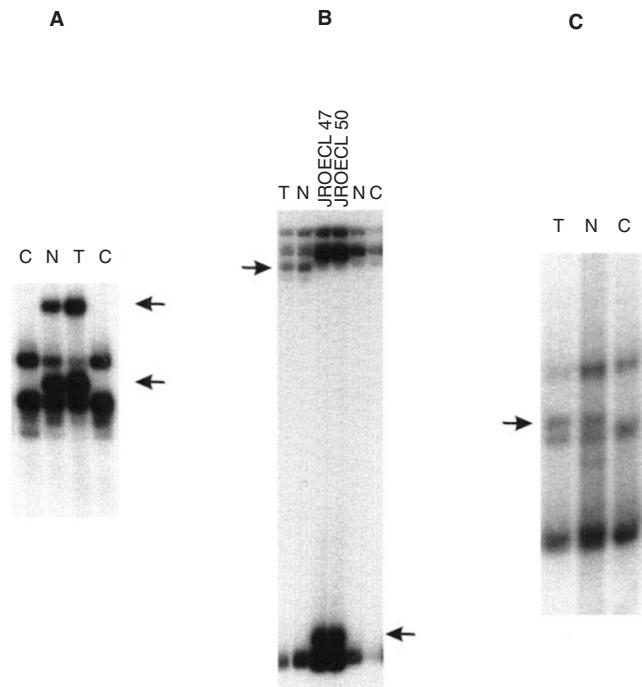
In 51 tumours LOH was determined with microsatellite markers that map on 16q22.1 where the E-cadherin gene is located. Markers tested were: D16S503, D16S265, D16S398 and D16S512. Markers were tested on 100 ng of tumour and normal DNA in a PCR reaction as described previously (Trapman et al, 1994). LOH was established by visual comparison of the intensity

of allelic bands obtained from tumour samples with those from normal DNAs.

## RESULTS

### PCR-SSCP

All 16 exons and exon-intron boundaries of the E-cadherin gene were analysed for genetic alterations in four cell lines, four xenografts and 49 tumour samples (45 primary tumours and four corresponding locoregional lymph node metastases). In total, 37 aberrant bands were detected throughout nine different amplicons. Cell line, xenograft and tumour DNA with aberrant SSCP patterns were compared with the amplification products of patient matched non-tumorous DNA. In most tumours the aberrant SSCP pattern was also present in the corresponding normal DNA (Figure 1). A tumour-restricted mobility shift was found in cell lines JROECL47 and JROECL50 in amplicon 3 (Figure 1B). Upon sequencing a one base-pair deletion in codon 120 (exon 3) was detected (Figure 2D and Table 1). Both cell lines were derived from the same primary tumour (SJ Darnton, personal communication), in which this mutation could not be detected. Sequence analysis of all the other SSCP band shifts revealed eight known polymorphisms and three new genetic alterations (Figure 2 A–C and Table 2). Furthermore, upon sequencing of amplicon 16 a discrepancy with the published sequence of intron 15 of the E-cadherin gene deposited in EMBL/GenBank database libraries (accession no. Z13009) was detected (5' intron15- ... cttgag-3' exon 16).



**Figure 1** PCR-SSCP analysis of E-cadherin amplicons 2 (A), 3 (B) and 4/5 (C) from three oesophageal adenocarcinoma specimens and two cell lines JROECL 47 and JROECL 50. Mobility shifts are identified by arrows. C, control DNA; T, tumour DNA; N, corresponding non-tumorous DNA

**Table 1** Tumour-restricted mutation in cell lines JROECL 47 and JROECL 50<sup>a</sup>

Amplicon	Site	Nucleotide change
3	Cdn 120	Deletion G: frameshift leading to stopcodon at codon 214 in exon 5

<sup>a</sup>Two cell lines derived from a primary tumour of one patient.

### LOH determination

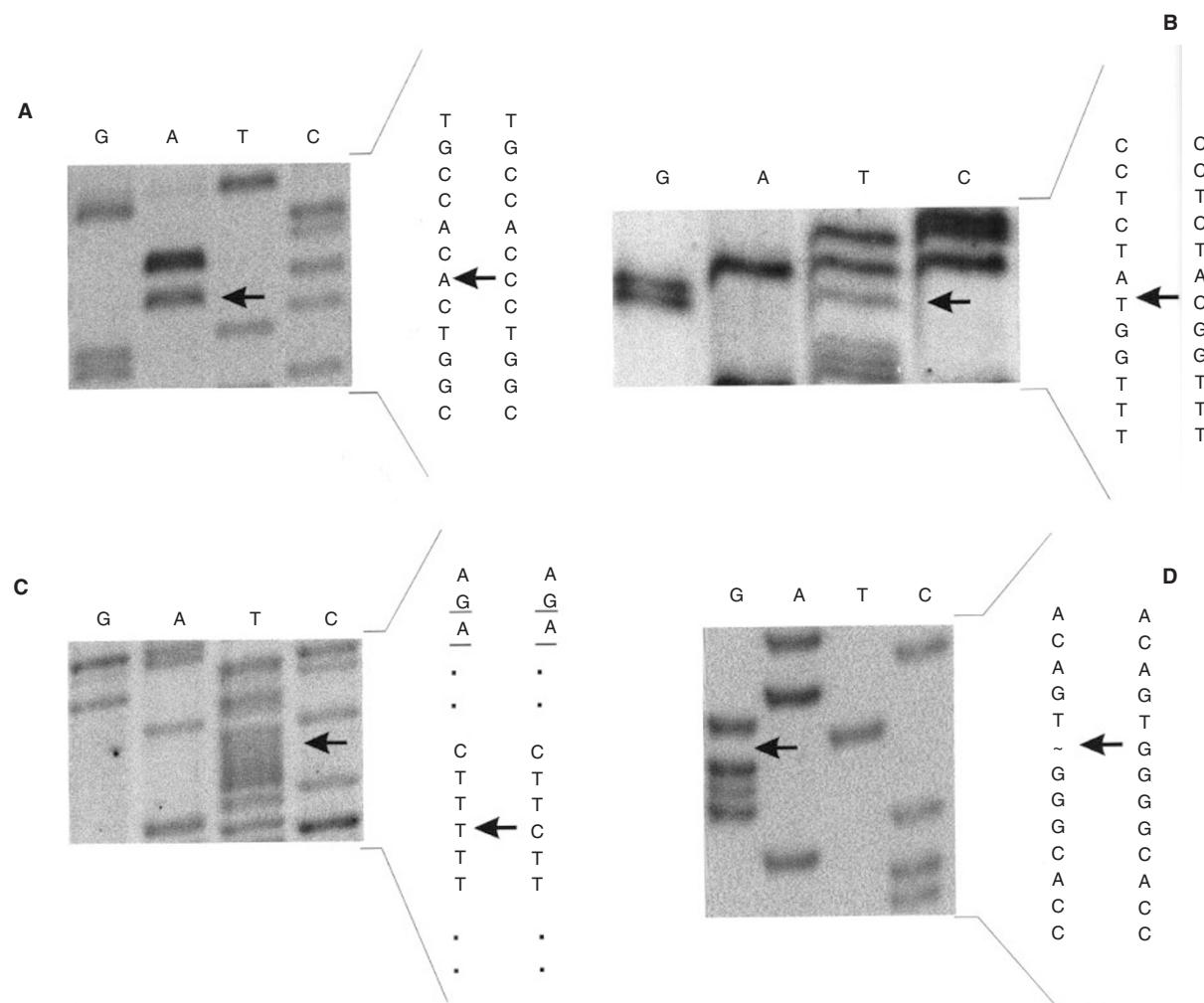
In Figure 3 examples are presented of the analysis of the four microsatellite loci in different tumour DNAs which showed allelic loss. Of the 48 informative cases, 31 (65%) showed LOH of at least one marker (Figure 4).

### DISCUSSION

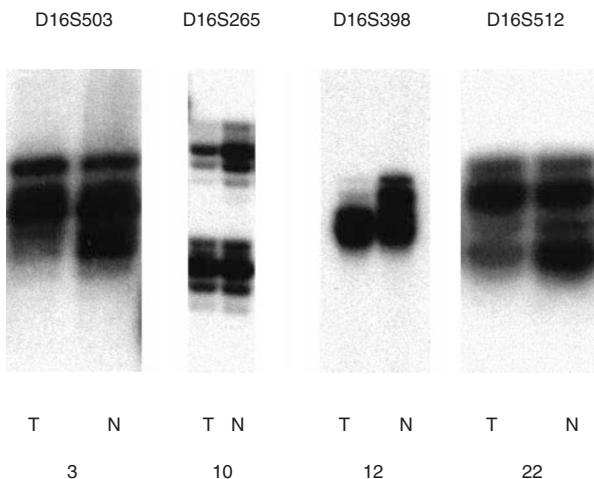
Reduced expression of E-cadherin is frequently seen in the majority of adenocarcinomas of the oesophagus and gastro-

oesophageal junction with or without Barrett's epithelium (Bongiorno et al, 1995; Swami et al, 1995; Bailey et al, 1998). However, E-cadherin immunoreactivity does not provide information about the function of the protein and gene integrity. For example, mutations in the E-cadherin gene leading to loss of adhesive potential but with normal cellular localization of the protein have been described (Becker et al, 1994; Oda et al, 1994). In this study we have analysed if genetic alterations in the E-cadherin gene are involved in the reduced expression of E-cadherin in adenocarcinomas of the oesophagus and gastro-oesophageal junction. In an earlier study, eight out of 49 tumours were analysed for expression of E-cadherin by means of immunohistochemistry. Seven out of eight of these adenocarcinomas showed reduced expression for E-cadherin (Krishnadath et al, 1997).

In our series of 57 adenocarcinomas of the oesophagus only one tumour restricted DNA alteration in the E-cadherin gene was detected. In two cell lines JROECL 47 and JROECL 50, derived from the same tumour, a one base-pair deletion was found leading to a premature stopcodon resulting in a truncated protein lacking



**Figure 2** Sequence analysis for the three tumours and cell line JROECL 47 with abnormally shifted PCR-SSCP bands as shown in Figure 1. Mutated sequence (left) and wild-type sequence (right) are shown at the right of each figure. Base-pair alterations are indicated by arrows. Splice recognition site is shown by the underlined characters in Figure 2C. For exon 2 (A) and exon 3 (B) we identified mis-sense mutations, also present in the normal control DNA of these patients. For amplicon 4/5 a base-pair change was observed in intron 4 (C). One frameshift mutation caused by a 1-bp deletion was detected for exon 3 (D) in the two cell lines JROECL 47 and JROECL 50



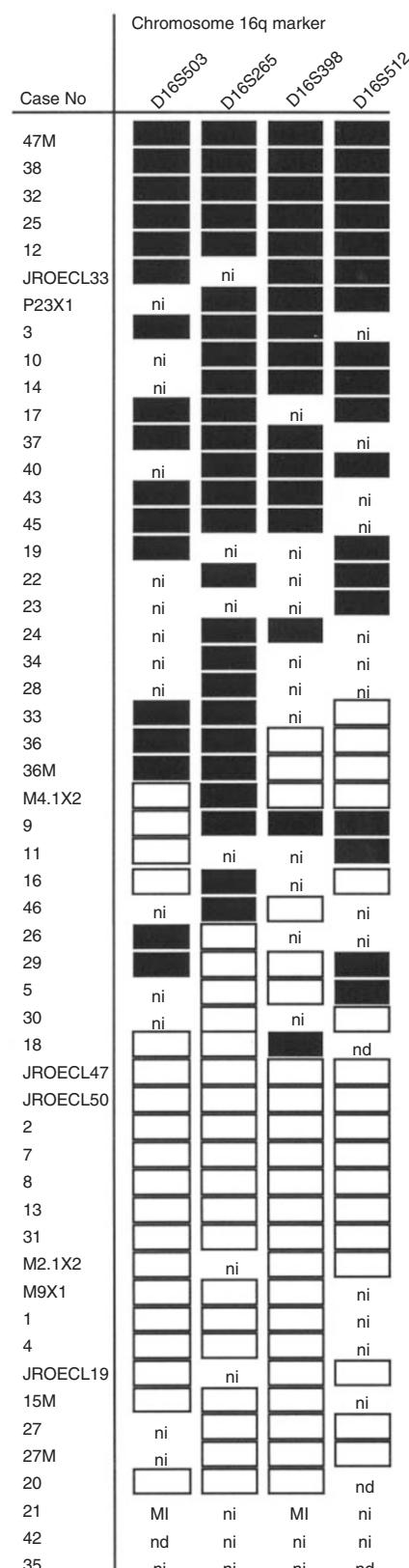
**Figure 3** Loss of heterozygosity in tumour 3 (D16S503), 10 (D16S265), 12 (D16S398) and 22 (D16S512). Loss of one allele is visible in the lane containing tumour DNA (T) compared with the lane containing normal DNA (N)

part of the extracellular binding region as well as the transmembrane and intracellular domain. So far, this mutation has not been reported in the literature. Both cell lines still have a non-mutated E-cadherin allele as demonstrated by the heterozygous exon 3 SSCP pattern indicating two different alleles. We were unable to demonstrate this mutation in the primary tumour. Obviously, the mutation was present in a minor subpopulation of the primary tumour or has developed during establishment of both cell lines.

PCR-SSCP analysis is a robust mutation detection procedure (Orita et al, 1989; Sheffield et al, 1993), indicated by the finding of eight known polymorphisms and three not yet described DNA alterations. However, we cannot rule out the possibility that mutations remained undetected by this procedure. All three new DNA alterations are also present in the patients' normal DNA. Two of these alterations lead to amino acid substitutions in the precursor sequence of the E-cadherin gene. Recently, germline mutations leading to truncated E-cadherin were identified in familial gastric cancer in New Zealand and Europe (Gayther et al, 1998; Guilford et al, 1998). Because no loss of the normal E-cadherin allele in the tumours occurred we consider these DNA alterations as polymorphisms. Furthermore, we have no evidence of a familial predisposition to oesophageal cancer in any of our patients.

Allelic loss at the E-cadherin gene locus 16q22.1 has been reported in 30–50% of breast, prostate and hepatocellular cancer (Tamura, 1997). Our data show LOH of this locus in two-thirds of the oesophageal adenocarcinomas. But the high percentage of 16q22.1 LOH without concomitant mutation of the remaining E-cadherin gene might point to another tumour suppressor gene on 16q involved in the genesis or progression of oesophageal adenocarcinomas. We cannot rule out the possibility that homozygous deletions are present in these carcinomas. Especially in DNA derived from resection specimens homozygous deletions can escape detection due to the contamination with non-tumorous DNA. However, all exons could be amplified with DNA derived from the cell lines and xenografts with exon spanning primers, which makes it unlikely that homozygous deletions have occurred.

Alternatively, if the high frequency of LOH at 16q22.1 truly reflects loss of one E-cadherin allele, this could point in the



**Figure 4** Patterns of allelic loss in oesophageal adenocarcinomas at the E-cadherin locus on chromosome 16q22.1. Closed rectangle, loss of heterozygosity; white rectangle, heterozygosity retained; ni, non-informative; MI, microsatellite instability; nd, no data obtainable. Note that 36M and 27M are lymph node metastases from primary tumours 36 and 27 respectively

**Table 2** Polymorphisms identified in the E-cadherin gene

Amplon	Site	Nucleotide change	Observed frequency <sup>a</sup> (57 tumours)	Literature <sup>b</sup>
1	Non-coding (5' UTR)	TCC→TGC	2/57	1
2	Cdn 30	CCT→ACT <i>Pro→Thr</i>	1/57	Unknown
3	Cdn 90	CGG→TGG <i>Arg→Trp</i>	1/57	Unknown
4/5	Intron 4 Intron 4	<u>gttagagaag</u> →..ac C→T 18 nt upstream exon 5	2/57 1/57	1 Unknown
11	Cdn 560	ACG→ACC <i>Thr→Thr</i>	2/57	1, 2
12	Cdn 632	CAC→CAT <i>His→His</i>	1/57	1, 2, 3
13	Cdn 692	GCT→GCC <i>Ala→Ala</i>	14/57	1, 2, 4
	Intron 12	T→C	12/57	1, 4
14	Cdn 751	AAC→AAT <i>Asn→Lys</i>	1/57	1, 2
16	Cdn 878	GGC→GGT <i>Gly→Gly</i>	1/57	3

<sup>a</sup>Splice recognition site is shown by the underlined characters. <sup>b</sup>Polymorphisms also reported by <sup>1</sup>Berx et al, 1996;  
<sup>2</sup>Guilford et al, 1998; <sup>3</sup>Risinger et al, 1994; <sup>4</sup>Soares et al, 1997.

direction of gene dosage effects as formerly proposed on the basis of experimental studies (Vleminckx et al, 1991). However, a previous study by Ilyas et al could not establish a correlation between allelic loss and immunohistochemical E-cadherin expression in colorectal cancers (Ilyas et al, 1997). Besides loss of one E-cadherin allele, other mechanisms leading to down-regulation of E-cadherin in oesophageal adenocarcinomas could also be involved.

Hypermethylation of the 5' CpG islands within the proximal promoter region of the E-cadherin gene has been found responsible for (temporary) down-regulation of E-cadherin in several cancers (Graff et al, 1998; Hiraguri et al, 1998; Saito et al, 1998). However, not only E-cadherin but also other members of the junctional complex have been shown to play a role in tumorigenesis. In several tumour cell lines showing impaired cell-cell adhesion and reduced expression of E-cadherin, mutations in  $\alpha$ -catenin and  $\beta$ -catenin were identified (Shimoyama et al, 1992; Kawanishi et al, 1995). A cytoplasmic and nuclear staining pattern of  $\beta$ -catenin has been observed in adenocarcinomas of the oesophagus in cases in which E-cadherin was reduced (Krishnadath et al, 1997; Bailey et al, 1998). These tumours could harbour mutated  $\beta$ -catenin, as was recently described for colon carcinomas and hepatocellular carcinomas (de La Coste et al, 1998; Iwao et al, 1998). Whether or not mutations in the catenins play a role in the pathogenesis of oesophageal cancer is presently unknown.

In conclusion, our results show that E-cadherin gene mutations are not involved in the subsequent progression of Barrett's epithelium to dysplasia and to adenocarcinoma of the oesophagus. Whether LOH at the E-cadherin locus contributes to the heterogenous expression of E-cadherin remains to be determined.

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