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E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers

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Compelling experimental evidence exists for a potent invasion suppressor role of the cell–cell adhesion molecule E-cadherin. In addition, a tumour suppressor effect has been suggested for E-cadherin. In human cancers, partial or complete loss of E-cadherin expression correlates with malignancy. To investigate the molecular basis for this altered expression we developed a comprehensive PCR/SSCP mutation screen for the human E-cadherin gene. For 49 breast cancer patients the occurrence of tumour-specific mutations in the E-cadherin gene was examined. No relevant DNA changes were encountered in any of 42 infiltrative ductal or medullary breast carcinoma samples. In contrast, four out of seven infiltrative lobular breast carcinomas harboured protein truncation mutations (three nonsense and one frameshift) in the extracellular part of the E-cadherin protein. Each of the four lobular carcinomas with E-cadherin mutations showed tumour-specific loss of heterozygosity of chromosomal region 16q22.1 containing the E-cadherin locus. In compliance with this, no E-cadherin expression was detectable by immunohistochemistry in these four tumours. These findings offer a molecular explanation for the typical scattered tumour cell growth in infiltrative lobular breast cancer.

Keywords: breast cancer/E-cadherin/invasion/truncation mutation/tumour suppressor gene

Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death (Berg and Hutter, 1995). Although analysis of allelic imbalance has yielded a number of putative tumour suppressor gene loci for sporadic breast cancer, so far only the p53 gene (TP53) clearly is mutated in a significant proportion of these tumours (Cornelis *et al.*, 1994; Devilee and Cornelisse, 1994). Loss of heterozygosity (LOH) on the long arm of chromosome 16 (16q) is one of the most frequently occurring genetic events in breast cancer (~50% of the informative cases). This indicates the presence of one or more tumour suppressor genes on 16q (Devilee and Cornelisse, 1994; Cleton-Jansen *et al.*, 1994; Rodriguez *et al.*, 1994; Tsuda *et al.*, 1994). For comparison, a similar frequency of LOH in sporadic breast cancer was reported for 17p harbouring the tumour suppressor gene TP53 (Devilee and Cornelisse, 1994). The E-cadherin gene is one of the candidate tumour suppressor genes on band 16q22.1, which is one of the smallest regions of deletion on this chromosome arm 16q (Cleton-Jansen *et al.*, 1994).

In epithelial cells, the transmembrane molecule E-cadherin is considered to be one of the key molecules for the formation of the intercellular junctional complex and for the establishment of cell polarization (Gumbiner *et al.*, 1988). The cytoplasmic tail of E-cadherin is linked via catenins to the actin cytoskeleton (Cowin, 1994), whereas the extracellular domain is involved in a molecular zipper mediating cell–cell adhesion (Shapiro *et al.*, 1995). E-cadherin cDNA transfection into invasive carcinoma cells demonstrated its strong invasion suppressor activity, at least *in vitro* (Frixen *et al.*, 1991; Vleminckx *et al.*, 1991). Activation of E-cadherin can cause growth retardation of tumour cells (Navarro *et al.*, 1991; Watabe *et al.*, 1994), and various junctional plaque proteins have been shown experimentally to act as tumour suppressor molecules (Tsukita *et al.*, 1993). The tumour suppressor gene *fat* in *Drosophila* has been found to be homologous to the cadherins (Mahoney *et al.*, 1991).

Immunohistological studies showed that the expression of E-cadherin is partially or totally lost in various human epithelial tumour types (Takeichi, 1993; Birchmeier and Behrens, 1994; Mareel *et al.*, 1994;). In breast cancer, reduced expression of E-cadherin is associated with invasiveness and loss of differentiation characteristics (Oka *et al.*, 1993). About 50% of the infiltrating ductal carcinomas, the predominant histological type of breast cancer (70%, Berg and Hutter, 1995), showed reduced E-cadherin expression in immunohistochemistry, whereas the less frequent infiltrative lobular carcinomas (6%) were in most cases completely E-cadherin negative (Gamallo *et al.*, 1993; Moll *et al.*, 1993; Rasbridge *et al.*, 1993).

These observations urged us to analyse the E-cadherin gene in human breast cancers. Here, we report the development of a PCR/SSCP assay for detection of E-cadherin gene variations in genomic DNA samples. For a series of 49 breast carcinomas this extensive mutation screen was combined with LOH analysis and immunohistochemistry. Our results provide strong evidence for the identification of E-cadherin as a tumour/invasion suppressor gene for sporadic infiltrative lobular breast carcinomas. We believe that the genomic mutation screen presented here will be valuable for the molecular analysis of E-cadherin down-regulation reported for many more carcinoma types.

Table I. Primers used for PCR amplification of E-cadherin exons

Exon No.	Sequence of S primer (5′→3′)[a]	Sequence of AS primer (5′→3′)[a]	Amplicon length (bp)	Annealing temp. (°C)	$MgCl_2$(mM)
1	(–53)tacgggggggcggtgctccgg	(+59)ctggggcgcggagcttgcgg	282	70	2
2	(–34)tcacccggttccatctac	(+229)caacctcctcttctttat	378	55	2
3	(–54)gctcttgtctttaatctgtc	(+75)gtaccaaggctgagaaacct	360	60	2
4 and 5	(–25)cttgttcctcatcttctttc	(+151)cccatcacttctccttagca	603[b]	55	2
6	(–18)ctcacttggttctttcag	(+60)aacctttgggcttggaca	246	55	3
7	(–37)agcttgtctaaaccttcatc	(+116)gcttagaccatcactgtatt	329	60	2
8	(–24)ttggttgtgtcgatctctct	(+70)cagtggtacccttagttcat	223	55	2
9	(–33)gtacttgtaatgacacatctc	(+36)tgccagtttctgcatcttgc	252	55	2
10	(–24)acttcattgtttctgctctc	(+41)aaccagttgctgcaagtcag	311	60	2
11	(–47)gttgtttgctggtcctattc	(+48)gaactagctaggaggtcgag	253	60	2
12	(–54)tggggattcattactgttgc	(+27)gcatggcagttggagcaaag	326	60	2
13	(–44)tttcctcccctggtctcatc	(+25)tgagtcacttgccagctgga	302	60	2
14	(–36)ctctcaacacttgctctgtc	(+22)agagatcaccactgagctac	209	60	2
15	(–67)catagccctgtgtgtatgac	(+33)cggatgctttggctttccac	248	60	2
16	(–57)agatgacaggtgtgcccttc	(+51)atttctgcatttcccagcac	315	60	2

[a]Positions of S (sense) and AS (antisense) primers are indicated by the number of nucleotides upstream (–y) or downstream (+y) from the nearest by exon (or stop codon for the AS primer of exon 16).
[b]This PCR product was cleaved by *Rsa*I in 221-bp and 382-bp fragments before SSCP (see Materials and methods).

Table II. Summary of E-cadherin gene variations in a set of 49 breast carcinomas (41 ductal, seven lobular and one medullary)

Tumour type	Codon No.	Amplicon[a]	Mutation or polymorphism	Tumour-restricted	Observed frequency
Ductal	Non-coding	1	TCC→TGC	No	1/49
Ductal	115	3	ACG→ACA *Thr→Thr*	No	1/49
Ductal	intron 4	4–5	gtagagaaag→ gtagagaaac	No	5/49
Lobular	261	6	**GAA→TAA** ***Glu→Stop codon***	**Yes**	1/49
Lobular	386	9	**GAG→TAG** ***Glu→Stop codon***	**Yes**	1/49
Lobular	399	9	**ACTGATG→ATG** ***4 bp del→Stop(codon 416)***	**Yes**	1/49
Lobular	504	10	**GAA→TAA** ***Glu→Stop codon***	**Yes**	1/49
Ductal	560	11	ACG→ACC *Thr→Thr*	No[b]	1/49
Ductal	692	13	GCT→GCC *Ala→Ala*	No[b]	13/49
Ductal	751	14	AAC→AAT Asn→Asn	No[c]	2/49

[a]Amplicons contain besides the exons, indicated by their number, flanking intron sequences and the whole intron 4 in the case of amplicon 4–5 (see also Table I).
[b]These polymorphisms were also reported by Risinger *et al.* (1994).
[c]This polymorphism was reported previously by Risinger *et al.* (1994) and by Becker *et al.* (1994).

Results

PCR amplification of the human E-cadherin gene

We recently isolated and characterized the full-size human E-cadherin gene (CDH1), spanning a region of 100 kb (Berx *et al.*, 1995). Via subcloning and partial sequence analysis we determined the sequence of all intron–exon boundaries. On the basis of these data we were able to develop 30 intron-complementary primers which enabled us to amplify all E-cadherin exons and the corresponding intron–exon junctions (Table I). The amplification for each amplicon was optimized for annealing temperature and for $MgCl_2$ concentration (Table I). In general, most PCRs worked fine with 2 mM $MgCl_2$ and with an annealing temperature of 55°C or 60°C. Only exon 1 was difficult to amplify due to its high GC-content. Therefore, we used a two-step PCR (annealing and reaction both at 70°C) on the basis of primers with a high melting temperature where DMSO was added at 5%.

Mutation screening of the human E-cadherin gene

To investigate the occurrence of any sequence variations in the genomic E-cadherin amplicons we sought a sensitive though economical detection technique allowing efficient screenings on a large scale. For these reasons, we preferred the single-strand conformation polymorphism (SSCP) analysis, known to be a reliable molecular scanning system for mutations and polymorphisms (Orita *et al.*, 1989). By radioactive labelling of the PCR products genetic changes should be detectable even in minor tumour parts, that might have undergone genotypic and malignant progression. To increase the detection efficiency we analysed all amplicons under two different non-denaturing gel conditions. All

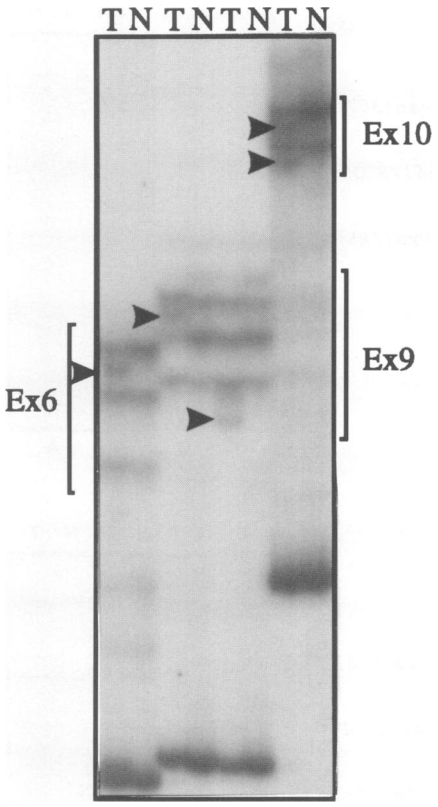

Fig. 1. PCR/SSCP analysis of E-cadherin exons 6, 9 and 10 from four lobular breast carcinomas. Exon 9 is analysed in the four central lanes. T, tumour DNA; N, corresponding normal tissue DNA.
Tumour-specific mobility shifts are indicated by arrowheads. Samples (2 μl) were loaded on a 5% polyacrylamide non-denaturing gel (ratio of acrylamide to bisacrylamide 38:0.8). Electrophoresis was at 3 W for 16 h at 8°C.

gels were run at controlled constant temperature to establish high reproducibility. The reliability of the analysis was shown by demonstrating in 20 blood samples of non-cancer patients several E-cadherin polymorphisms, previously reported by others (Risinger *et al.*, 1994; our data not shown).

Breast tumour analysis for E-cadherin mutations and LOH

We tested 49 breast tumour samples consisting of 41 infiltrative ductal carcinomas, seven infiltrative lobular carcinomas and one medullary carcinoma. By analysis of all E-cadherin exons using the two different SSCP gel conditions, 10 different mobility shifts were identified. Comparison of the aberrant tumour DNAs with amplified blood DNA from the same patients revealed that only four of the mobility shifts were tumour-restricted and all of them were found in samples of infiltrative lobular carcinomas (Figure 1). Direct sequence analysis showed that indeed six out of ten sequence variations were E-cadherin germline polymorphisms without influence on the amino acid sequence (Table II). On the other hand, three tumour-restricted band shifts were caused by nonsense mutations affecting, respectively, codon 261 in exon 6, codon 386 in exon 9 and codon 504 in exon 10 (Table II and Figure 2). The last tumour-restricted bandshift was due to a 4 bp deletion in exon 9, resulting in a stop codon downstream at codon 416 (Table II, Figures 1 and 2). It is noteworthy that DNA extracted from two lymph node metastases of the latter tumour did show the same exon 9 bandshift in highly enriched amounts. This is due to the fact that the tissue block of this particular primary tumour contained only 30% tumour cells, whereas the lymph node metastases had less contamination with non-tumour tissue and contained 70–80% tumour cells. This was assessed by estimation of the percentage of tumour cells on haematoxylin–eosin-stained sections of the tumour tissue blocks. For the four lobular breast tumour samples harbouring truncation mutations in E-cadherin and for the two lymph node metastases mentioned we also found consistent LOH for 16q22.1 bearing the E-cadherin locus (exemplified in Figure 3, left panels). The three other infiltrative lobular tumours without detectable E-cadherin mutations did not show LOH on 16q22.1 (Figure 3, right panels). Loss of heterozygosity on chromosomal band 16q22.1 was also found in 27 (66%) out of the 41 infiltrative ductal breast tumour samples that we have screened in this study. However, E-cadherin mutations could not be detected in these tumours.

Immunohistological staining

All seven lobular carcinomas and a subset of 14 ductal carcinomas were tested for expression of the E-cadherin protein by immunohistochemical staining with the HECD-1 monoclonal antibody, recognizing an extracellular epitope on human E-cadherin (Figure 4). There was some variation in staining intensities when the ductal carcinomas were compared with each other. In 10 out of 14 ductal carcinoma samples normal ducts were present that could serve as internal controls for positive staining. In nine cases, staining intensity of the tumour cells was not decreased as compared with that of the normal ductal epithelial cells, whereas in one case the tumour cells showed a slightly weaker staining. Like the normal duct cells the tumour cells showed positive plasma membrane-associated staining (Figure 4A), although in most cases a very faint cytoplasmic staining and some intercellular staining heterogeneity could be observed. Of the lobular carcinomas, six did not show staining in the tumour cells, whereas the normal ducts and lobules were clearly positive (Figure 4B). Tumour cells of one lobular carcinoma showed positive staining all over their surface, but the typical honeycomb staining pattern was absent in this scattered tumour type (Figure 4C). In this tumour, BT655, neither E-cadherin mutations nor LOH on 16q22.1 were detected (Figure 3). Hence, E-cadherin expression was absent in all four tumours with truncation mutations plus LOH on 16q22.1. Two additional lobular tumours in which neither a mutation in the E-cadherin gene nor LOH on 16q22.1 could be detected were nevertheless negative for E-cadherin protein expression.

Discussion

The data reported here consolidate the evidence for an important role of E-cadherin as an invasion/tumour suppressor in particular carcinomas. In total, we examined 49 breast carcinomas, of which seven were infiltrative lobular carcinomas, on the genomic DNA level for E-cadherin mutations. Besides polymorphisms only four

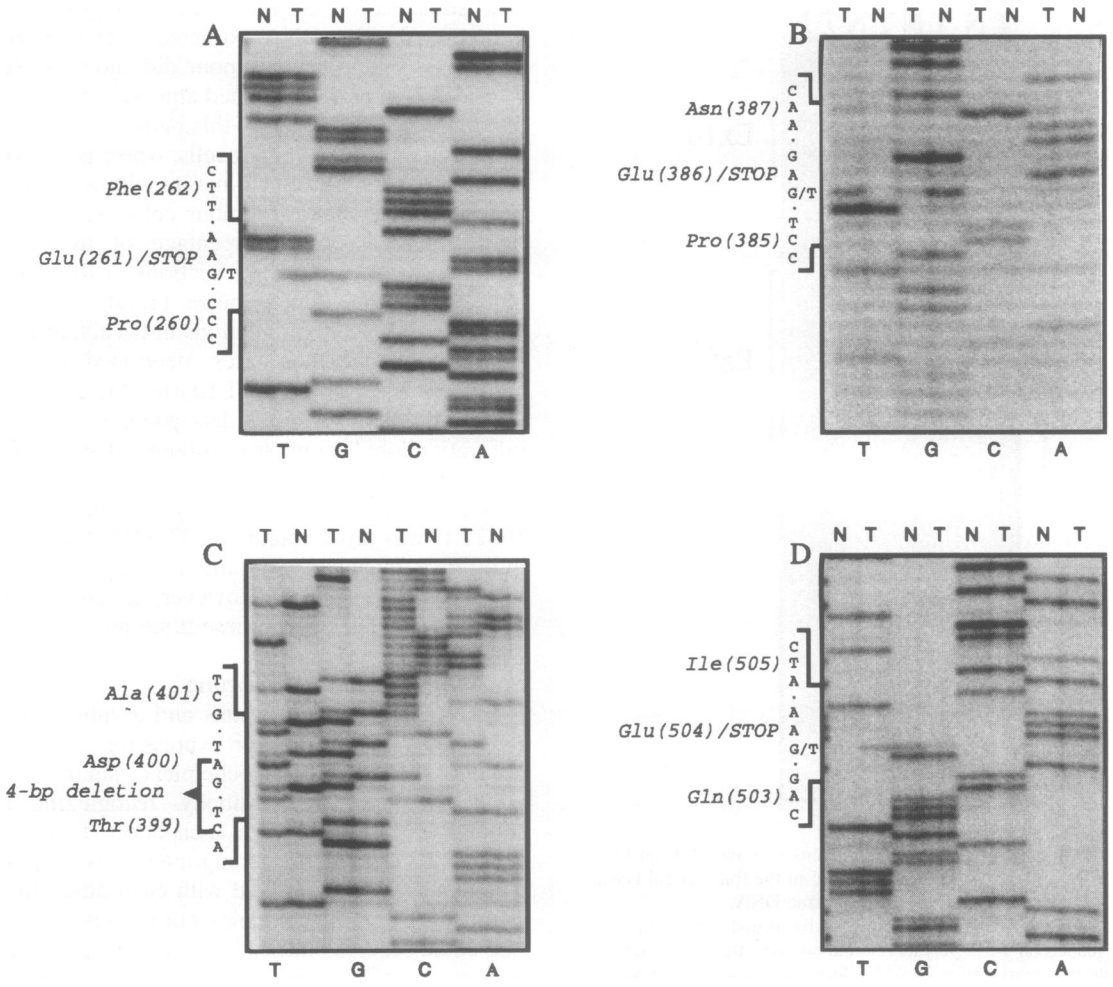

Fig. 2. Sequencing for four lobular breast samples of abnormally shifted PCR/SSCP bands as shown in Figure 1. For exon 6 (**A**), exon 9 (**B**) and exon 10 (**D**), we identified point mutations resulting in stop codons. One frameshift mutation caused by a 4-bp deletion was detected for exon 9 (**C**). Codon numbers are indicated between brackets. DNA of normal tissues (N) is compared lane by lane with the tumour DNA (T).

tumour-restricted mobility shifts were detected by our new E-cadherin PCR/SSCP analysis and these four tumours were all of the infiltrative lobular type (Table II and Figure 2). Infiltrative lobular carcinomas of the breast generally lack E-cadherin expression (Gamallo *et al.*, 1993; Moll *et al.*, 1993; Rasbridge *et al.*, 1993), and show a histological growth pattern fully compatible with early loss of this major cell–cell adhesion molecule. Indeed, whereas most infiltrating ductal carcinomas consist of cohesive groups of tumour cells, infiltrating lobular carcinoma cells often infiltrate as single cells, sometimes grouped in so-called 'Indian files' (Figure 4). For four out of six lobular breast carcinomas, found by us to lack E-cadherin expression, we identified both LOH of 16q22.1 harbouring the E-cadherin locus and tumour-restricted truncating mutations in the remaining allele. Hence, the E-cadherin gene fulfils the criteria of classical tumour suppressor genes, inactivated by two genetic hits (Knudson, 1985).

Sequence analysis for three PCR products with aberrant mobility, covering E-cadherin exons 6, 9 and 10, revealed for all cases a Glu→stop codon mutation (Table II and Figure 2). For another exon 9 mobility shift we identified a 4 bp deletion resulting in a frameshift and a generation of a stop codon at codon 416. Recently, Kanai *et al.* (1994) reported an E-cadherin mutation study on 22 invasive lobular breast carcinomas. The only mutation identified was an Asn→Ser missense mutation at codon position 315 (exon 7), found in two tumours. We were unable to detect such change in our samples using a variety of SSCP gel conditions besides those detailed here. A possible reason why Kanai *et al.* (1994) did not identify E-cadherin nonsense mutations may be the fact that they analysed only exons 5, 6, 7 and 8, whereas we screened all 16 exons.

Our immunohistological analysis confirmed that E-cadherin expression was rarely detectable in lobular breast carcinomas, in contrast to ductal breast carcinomas where in most cases no or only partial loss of E-cadherin expression is found (Figure 4) (Gamallo *et al.*, 1993; Moll *et al.*, 1993; Rasbridge *et al.*, 1993). There was variation in E-cadherin staining intensity between cases. However, staining intensity in the tumour cells was nearly always similar to that in normal ductal epithelial cells present in the same tissue section. From this we conclude tentatively that the variation in staining intensity for most cases of ductal carcinoma has no biological significance, but is most likely due to technical problems such as fixation of the tissue blocks. Interestingly, soluble E-cadherin

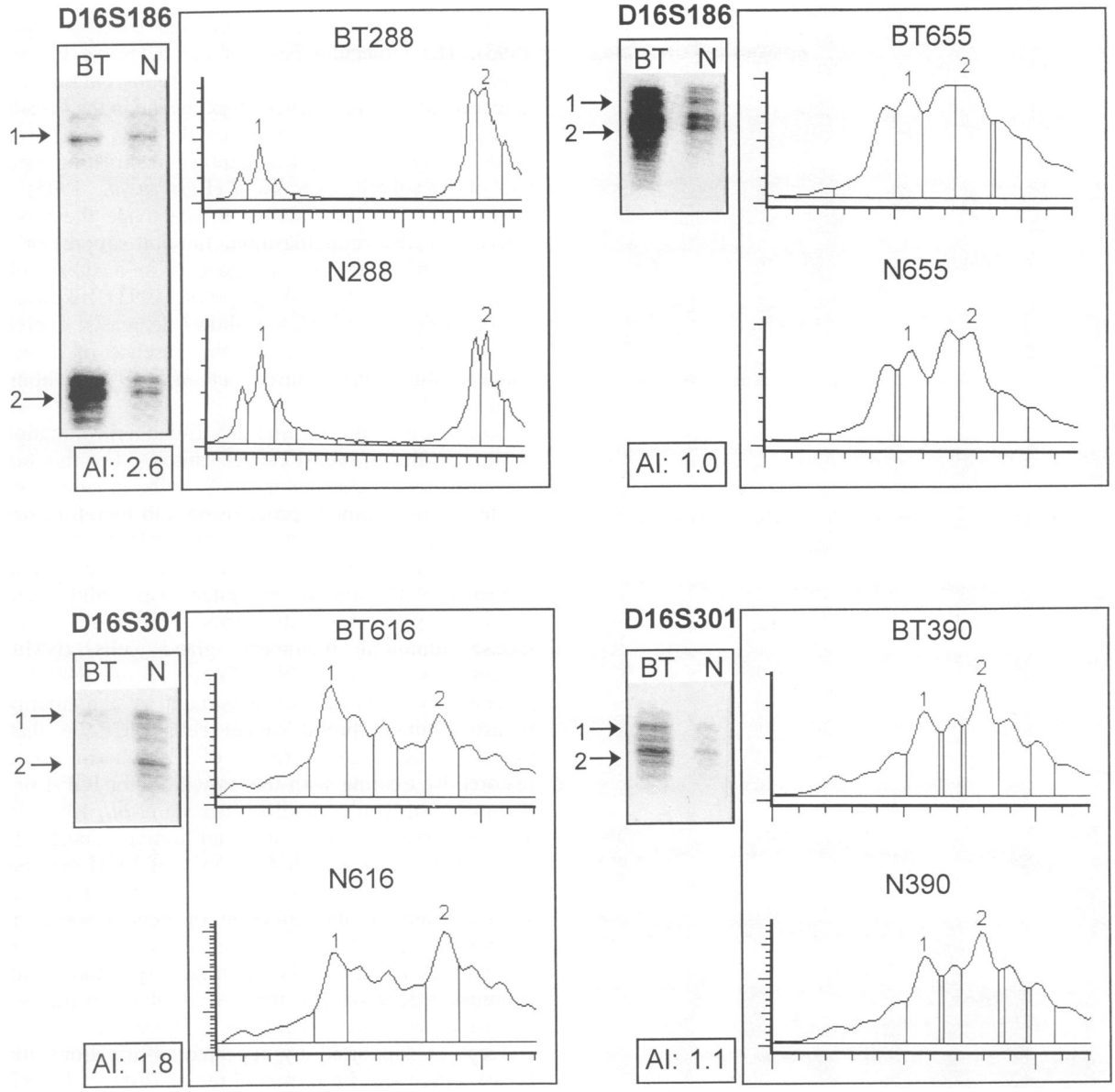

Fig. 3. Autoradiographs and PhosphorImager traces of PCR products obtained with the polymorphic microsatellite markers D16S186 and D16S301 when tested on matched samples of breast tumour DNA (designated BT) and DNA from normal cells (N). Arrows and numbers 1 and 2 indicate the two alleles. AI, allelic imbalance factor (see Materials and methods). Tumours BT288 and BT616 show LOH on 16q22.1 and have a nonsense mutation in the E-cadherin gene. BT655 and BT390 show neither LOH nor mutations in the E-cadherin gene.

fragments have been reported in the medium of the human breast cancer cells MCF-7 and in the serum and urine of cancer patients (Damsky *et al.*, 1983; Katayama *et al.*, 1994a,b). These soluble forms are probably produced by endoproteolytic degradation as they show a fairly constant molecular mass of ~80 kDa. They are able to disrupt E-cadherin-mediated cell–cell adhesion *in vitro* (Wheelock *et al.*, 1987). The premature chain terminations found by us in lobular breast samples would result in secreted E-cadherin fragments smaller than 80 kDa, but it is quite possible that also these fragments interfere with the proper function of normal E-cadherin molecules. This may result in peritumoral adhesion disruption events and in enhanced invasiveness of the tumour cells secreting such peptides. This hypothesis is supported by the finding that even decapeptides containing the HAV homophilic recognition sequence of E-cadherin are able to disturb proper cell–cell adhesion (Blaschuk *et al.*, 1990). The four mutations described here are all carboxy-terminal of the HAV sequence. The tumour cells are expected to secrete amino-terminal fragments, recognizable by the HECD-1 antibody used. The absence of positive staining in these four tumours may be due to instability or draining of the antigen.

Our data strongly suggest that premature stop codons in combination with loss of the non-mutated allele are the cause of the typical scattered cell growth in about half (four out of seven) of lobular breast cancers. Whether the observed E-cadherin mutations may serve as a prognostic marker for lobular breast cancer will depend on further studies on larger tumour series. To explain the characteristic histology in the other lobular breast samples without detectable E-cadherin mutations, we should discriminate between lobular tumours showing E-cadherin protein

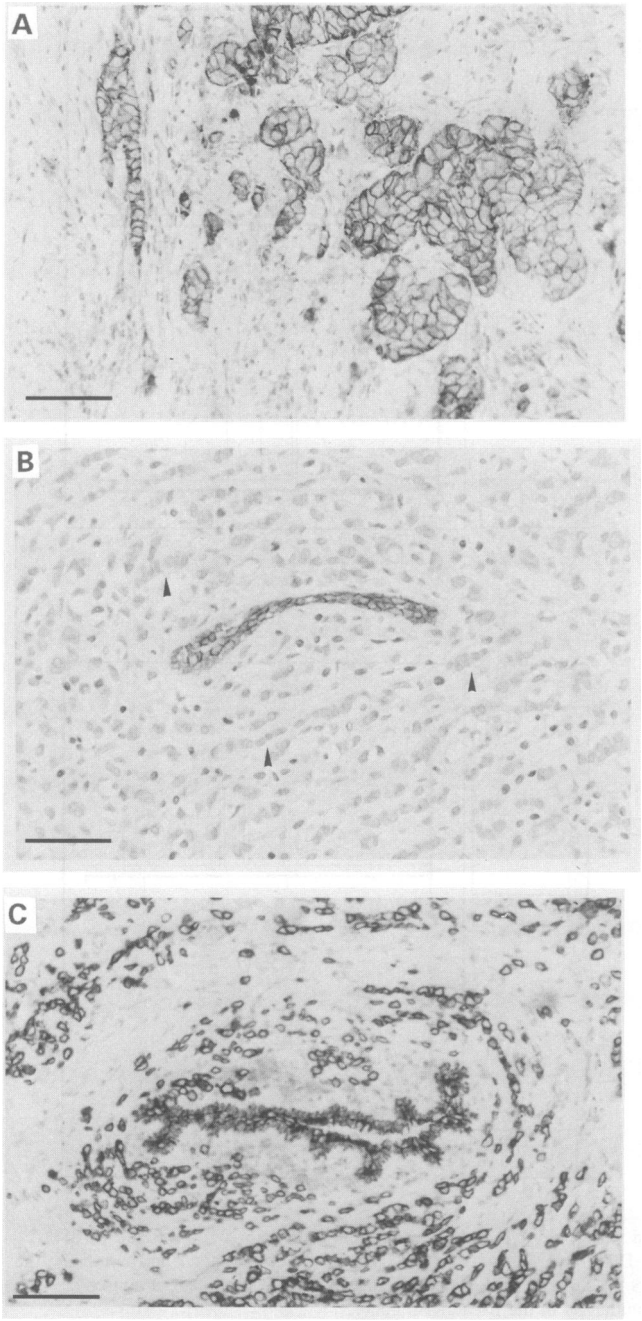

Fig. 4. Breast carcinomas, stained for E-cadherin expression using monoclonal antibody HECD-1. (**A**) Invasive ductal carcinoma: positive for E-cadherin expression. The tumour cells show clear plasma membrane staining at the cell surface. (**B**) Invasive lobular carcinoma: negative for E-cadherin expression. A pre-existing compressed duct (in the centre) is positive for membrane staining; the surrounding tumour cells are negative. Notice the 'Indian file' growth pattern (arrowheads). (**C**) Invasive lobular carcinoma: positive for E-cadherin expression. The tumour cells, as well as the normal duct cells in the centre, show clear plasma membrane staining. However, cells from the duct show evident polarization (lack of staining at the luminal side), whereas non-polarized tumour cells are stained all over their surface. Magnification bar = 100 μm.

expression (one case here; Figure 4C) and tumours lacking E-cadherin expression. For the former case, it will be interesting to look at α-catenin expression, which is also frequently lost in this tumour type (Ochiai *et al.*, 1994). Defects in catenins will result in non-polarized expression of E-cadherin (cf. Figure 4C), in less-organized cell junctions, and in weaker intercellular adhesion (Kemler, 1993). The molecular basis of the defective E-cadherin expression in the latter tumours remains enigmatic. The detection of a 5′ high-density CpG island in the E-cadherin gene suggests that transcriptional down-regulation by DNA methylation might be another inactivating mechanism of E-cadherin expression (Berx *et al.*, 1995). CpG methylation has been shown to inactivate the promoter activity of the retinoblastoma tumour suppressor gene and contributes to the oncogenesis in a subset of the retinoblastomas (Ohtani-Fujita *et al.*, 1993). Likewise, the tumour suppressor BRCA-1 shows decreased expression in sporadic breast cancer in the absence of detectable somatic mutations (Futreal *et al.*, 1994; Thompson *et al.*, 1995).

So far, we could not find any E-cadherin mutations in infiltrative ductal breast cancer samples. For this tumour type irreversible changes in the E-cadherin gene could be a late event in tumour progression and therefore hard to detect, even by radioactive PCR/SSCP. Reversible and focal down-regulation of E-cadherin expression under influence of the tumour ecosystem was demonstrated in *in vivo* experiments with various cell types including mouse tumoural mammary gland cells (NMuMG) (Vleminckx *et al.*, 1991; Mareel *et al.*, 1993). This reversibility is further demonstrated by the finding for invasive human breast cancer cells MCF-7/6 that the invasion suppressor activity of E-cadherin could be restored by treating with the growth factor IGF-I or with the anti-oestrogen tamoxifen (Bracke *et al.*, 1993, 1994). The observation that also infiltrative ductal breast carcinomas show a high frequency of LOH on 16q22.1 (27 out of 41 tumours in this study) (Cleton-Jansen *et al.*, 1994), points in the direction of gene dosage effects formerly proposed on the basis of experimental studies (Vleminckx *et al.*, 1991). Alternatively, another tumour suppressor gene on 16q may be involved in the genesis or progression of ductal breast carcinomas. This latter concept is supported by repeated observations that in breast tumours a chromosomal region distal to E-cadherin is often involved in LOH, whereas markers on 16q22.1, the chromosomal band that contains the E-cadherin gene, show retention of heterozygosity (Cleton-Jansen *et al.*, 1994; Tsuda *et al.*, 1994; Skirnisdottir *et al.*, 1995). Also, phosphorylation of β-catenin or reduced expression of either α-catenin or plakoglobin (γ-catenin) may be important in the acquisition of the invasive phenotype by breast cancer cells, despite continued expression of E-cadherin (Sommers *et al.*, 1994; Takayama *et al.*, 1994).

LOH for the E-cadherin chromosomal location 16q22.1 has been detected in many carcinoma types besides breast cancer (Tsuda *et al.*, 1990; Bergerheim *et al.*, 1991; Sato *et al.*, 1991). Regarding optimum development of therapeutic strategies it is of high priority to determine in what kind of epithelial tumours E-cadherin is functionally inactivated by irreversible mutations. The mutations reported so far for human tumours and tumour cell lines are summarized in Figure 5. Only three missense mutations and one frame shift were detected for the E-cadherin gene in a series of 135 carcinomas of endometrium and ovary (Risinger *et al.*, 1994). Interestingly, E-cadherin gene mutations, often resulting in exon skipping, were found in 50% (13 out of 26) of diffuse-type human gastric

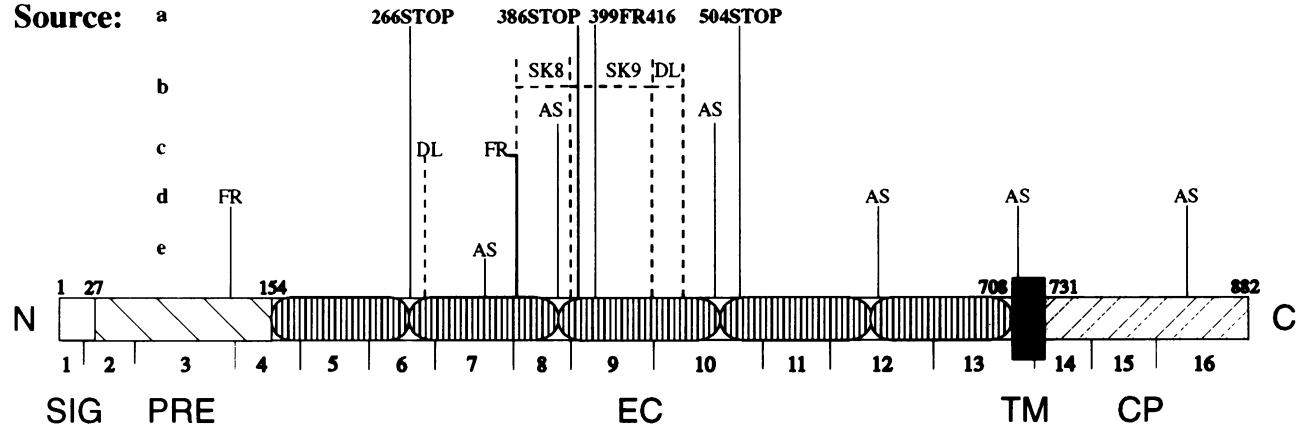

Fig. 5. Schematic representation of non-silent mutations reported so far for the human E-cadherin. N, amino-terminus; C, carboxy-terminus; SIG, signal peptide; PRE, precursor sequence; EC, extracellular domains with Ca^{2+}-binding motifs; TM, transmembrane domain; CP, cytoplasmic domain. Numbers on top of the protein denote codon numbers at domain borders. Numbers underneath the protein denote the encoding exons. Line **a** summarizes the mutations described for infiltrative lobular breast cancer in the present report (see Table II; numbers point at codons mutated; Stop indicates a nonsense mutation; FR416 indicates a frameshift resulting in a stop codon at position 416). Other mutations are depicted as: AS, amino acid substitution; DL, deletion; FR, frameshift by deletion or insertion; SK, skipping of exon. They comprise mutations in gastric carcinomas (line **b**) (Becker *et al.*, 1993, 1994), gastric carcinoma cell lines (line **c**) (Oda *et al.*, 1994), cancers of endometrium and ovarium (line **d**) (Risinger *et al.*, 1994) and lobular breast cancers (line **e**) (Kanai *et al.*, 1994).

carcinomas, but not in intestinal-type gastric carcinomas (Becker *et al.*, 1993, 1994). For one gastric tumour, it was shown that the wild-type allele was lost, whereas the mutated allele was retained. Also studies on gastric carcinoma cell lines revealed E-cadherin mutations (Oda *et al.*, 1994). It is noteworthy that up to now, no nonsense mutations were reported whereas three out of four mutations detected by us were of this type. This may reflect a particular aetiology for the present lobular breast carcinomas.

It is also clear from Figure 5 that the E-cadherin mutations so far reported are scattered over 9 out of 16 exons, although there is a tendency of accumulation in exons 6–10 encoding extracellular domains of E-cadherin. Hence, scanning the whole gene for tumour-related mutations is still required, especially when investigating new tumour types. To facilitate such analysis on a large-scale basis we present here a reliable and simple genomic mutation screen comprising PCR/SSCP with E-cadherin intron-specific amplimers. On the basis of the cloned human E-cadherin gene (Berx *et al.*, 1995), 30 primers were synthesized to amplify the 16 exons with corresponding intron junctions. All PCRs were optimized and tested for PCR/SSCP. Tests for the sensitivity and reliability of this mutation screen will be reported elsewhere, together with a handy multiplex set-up of this assay. Our approach has two advantages in comparison with RT–PCR/SSCP and RT–PCR/sequencing as applied by, respectively, Risinger *et al.* (1995) and Becker *et al.* (1994). First, the quality of the starting material is less critical, and second, mutations located in splice recognition sites may be immediately identified.

Materials and methods

DNA isolation

Selection of breast carcinomas was described previously in Cleton-Jansen *et al.* (1994). Breast tumour DNA was isolated from freshly frozen tissue blocks as described (Devilee *et al.*, 1991). The tumour tissue samples contained at least 50% tumour cells to assure a reliable detection of LOH. Corresponding constitutional DNA was isolated from peripheral blood lymphocytes.

Immunohistochemical staining

Immunohistochemistry was performed on 5 μm sections from paraffin-embedded tumour tissue blocks with monoclonal antibody HECD-1 (Zymed Laboratories, South San Francisco, CA) using the antigen retrieval protocol described in Hazelbag *et al.* (1995).

LOH determination

Loss of heterozygosity was determined as described (Cleton-Jansen *et al.*, 1994), with the following polymorphic microsatellite markers on chromosomal band 16q22.1: D16S186, D16S265, D16S301, D16S318 and D16S398. Quantification of the allele intensities was determined on a Molecular Dynamics PhosphorImager 445 SI. Molecular Dynamics ImageQuant software was used for the quantification of PCR products. The allelic imbalance (AI) factor is the quotient of the peak ratios from, respectively, tumour and constitutional DNA. An AI factor ≤ 1.3 is interpreted as retention of heterozygosity, whereas an AI >1.5 is considered as LOH.

PCR/SSCP analysis

PCR primers were synthesized on an Applied Biosystems 381A DNA synthesizer. The primers used in the SSCP analysis are shown in Table I. Genomic DNA was used at 250 ng per 25 μl reaction mixture containing 2–3 mM $MgCl_2$ (see Table I), 200 μM of dATP, dGTP and dTTP, 20 μM dCTP, 0.5 μCi [α-^{32}P]dCTP (Amersham), 25 pmol of each primer and 0.2 U *Taq* polymerase. Each PCR was overlaid with mineral oil and after an initial incubation at 95°C for 2 min, PCR was performed for 35 cycles consisting of 94°C for 30 s, 55–70°C (see Table I) for 30 s and 72°C for 45 s on a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Cetus) (Table II). To reduce the size of the amplicon spanning exon 4–5 (Table I), we digested the PCR product with *Rsa*I by adding 2 U enzyme directly to the PCR mixture and incubating for 2 h at 37°C. This yielded 221 bp and 382 bp fragments, specific for, respectively, exon 4 and exon 5. A 5 μl aliquot of PCR product was mixed with 5 μl of formamide dye (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 96°C for 2 min and snap-cooled on ice. Aliquots of 2 μl were analysed simultaneously on two non-denaturing polyacrylamide gels (5% acrylamide with 2% cross-linking), either containing 5% glycerol and run at 20°C, or lacking glycerol and run at 8°C. Electrophoresis was performed in 0.5× TBE on vertical gels (530×210×0.4 mm) in a water-jacketed, temperature-controlled Pharmacia Macrophor apparatus at 3 W for 16 h. Gels were dried on 3MM Whatman paper and autoradiography was performed using Hyperfilm (Amersham), generally for 6–12 h at room temperature without intensifying screen.

Sequencing of PCR products

Bands with abnormal mobility were excised from the Whatman paper, placed in 150 µl double-distilled H_2O, and incubated for 30 min at 60°C followed by 15 min at –70°C. This was repeated three times. A 10 µl aliquot of the supernatant was used as PCR template for 35 cycles as described above. A 5 µl aliquot of the PCR was then incubated with 1 U exonuclease I and 1 U shrimp alkaline phosphatase to remove excess of primers and dXTPs (Sequenase™ PCR Product Sequencing Kit, Amersham). Sequences of both strands were determined by modified T7 polymerase (Sequenase 2.0, USB) using either one of the original PCR primers.

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