Virchows Arch (1997) 430:23–28

ORIGINAL ARTICLE

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E-Cadherin (E-cad) expression in duct carcinoma in situ (DCIS) of the breast

Received: 11 June 1996 / Accepted: 12 August 1996

Abstract E-cadherin (E-cad) is an epithelial cell-cell adhesion molecule whose loss or reduced expression is associated with a more invasive tumour phenotype. Ninety-six cases of screen detected pure ductal carcinoma in situ (DCIS) were analysed immunocytochemically for expression of E-cad using the HECD-1 mouse monoclonal antibody. The in situ component in each case was classified on the basis of cytonuclear grade, extent of necrosis, Van Nuys classification and a newly devised Cardiff classification. The amount of E-cad expression was assessed semi-quantitatively using an intensity distribution method. There is significantly more expression of E-cad in well-differentiated DCIS when compared with poorly differentiated DCIS and this finding is highly significant ($P<0.001$) irrespective of the classification system used. These findings suggest that progressive loss of E-cad expression may occur at an early stage of breast cancer development.

Key words Adhesion molecules · E-cadherin · Duct carcinoma of breast · Breast

Introduction

E-cadherin (E-cad) is a molecule which mediates cell-to-cell cohesion in epithelia. Normal mammalian epithelial cells express E-cad in order to maintain cell-to-cell integrity. Over the past few years identification of several intercellular adhesion molecules has led to a greater understanding of their role, both in normal cellular physiology as well as in the pathophysiology of various disease processes. It is now increasingly recognised that adhesion molecule dysfunction plays a pivotal role in cancer progression and tumour metastasis [13]. This recognition of the role of adhesion molecules has opened up a potential new area of diagnosis and therapy of malignant disease. Manipulation of adhesion molecule function either at the genetic level or by monoclonal antibodies may open the door for novel therapeutic regimes to prevent cancer progression and tumour metastasis.

One of the features of malignant epithelial cells is loss of cohesion and this has been shown to be due to down regulation of E-cad expression [3]. Antibodies have been raised to the E-cad molecule which are capable of disrupting cell-to-cell epithelial adhesion in tissue culture [10]. Monoclonal antibodies are also available capable of detecting the presence of E-cad molecules on the surface of cells in formalin-fixed, paraffin-embedded tissues. Studies on the expression of E-cad in infiltrating carcinoma of the breast have shown reduced expression of E-cad associated with high histological grade, histological type (with consistent loss in lobular carcinoma) and with nodal metastases [7, 14, 16, 17, 22, 25]. It is not known if loss of E-cad expression is an early event in breast cancer progression and there is little information on E-cad expression in intraductal breast carcinoma. We have examined the expression of E-cad in relation to differentiation in duct carcinoma in situ (DCIS) of the breast.

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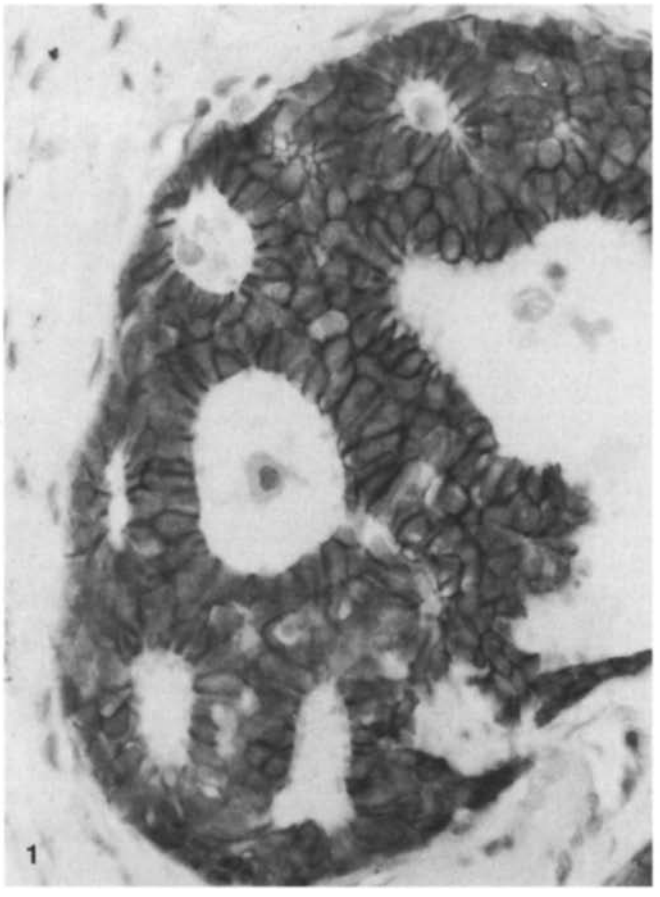
Materials and methods

The monoclonal anti-E-cad antibody (HECD-1) capable of binding E-cad in formalin-fixed, paraffin-embedded tissue was used in this study [20].

Ninety-six cases of pure intraduct carcinoma of the breast were retrieved from the files of the University Hospital of Wales (UHW). Five micron sections were cut and stained with routine haematoxylin and eosin. The DCIS was graded using cytological



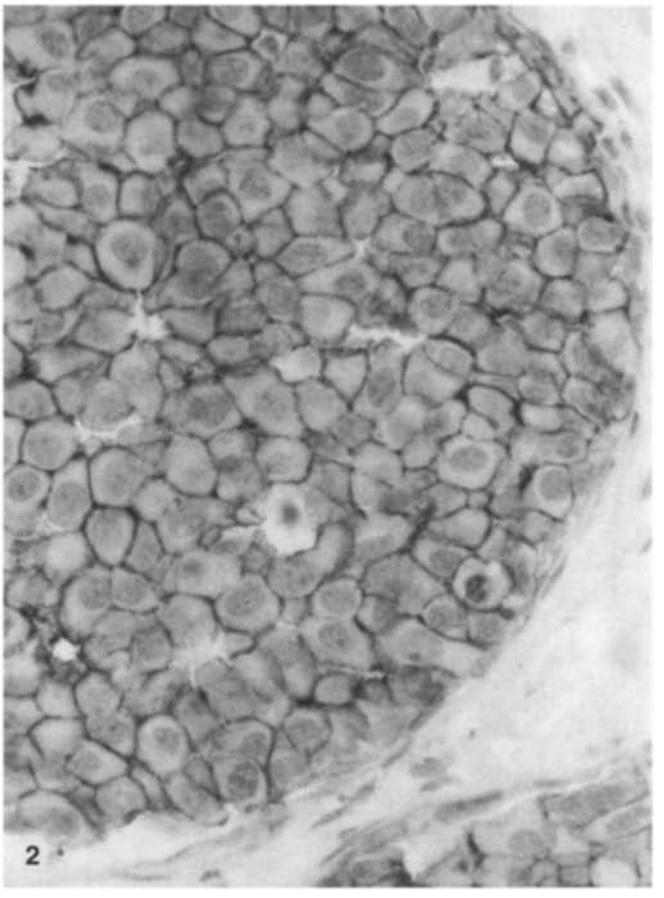


Fig. 1 An example of well-differentiated cribriform ductal carcinoma in situ (DCIS) (nuclear score 1, necrosis score 1) showing strong (3+) immunopositivity for E-cad in 70% of the cell membranes leading to a total ID score of 210 (× 400)

Fig. 2 An example of poorly differentiated DCIS (score 3 for nuclear pleomorphism, 3 for necrosis). There is 1+ immunopositivity in 20% of the cell membranes present giving an ID score of 20 (× 400)

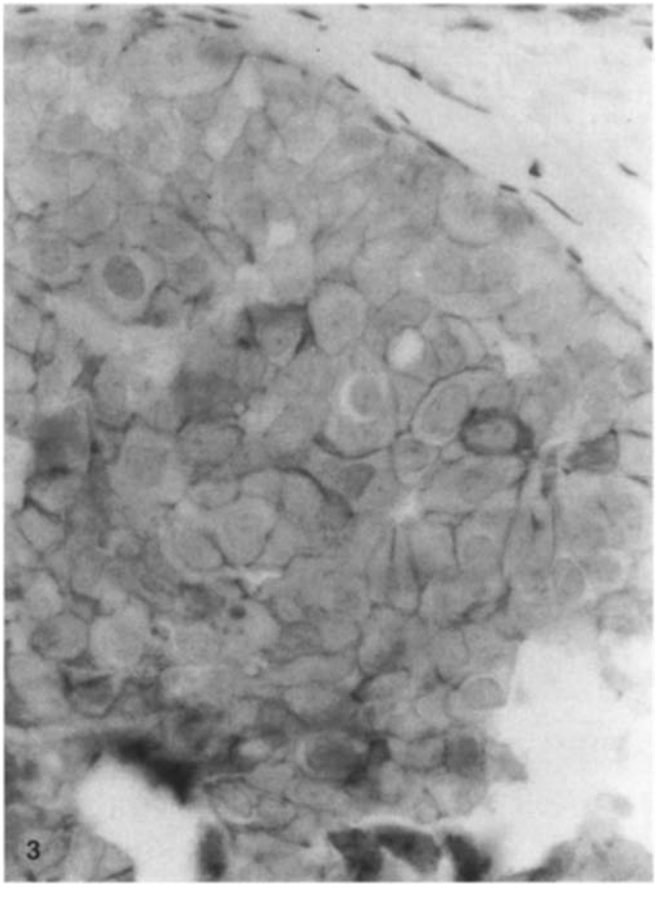


Fig. 3 An example of poorly differentiated DCIS (cytonuclear pleomorphism score 3, necrosis score 3) showing uniform strong (3+) membrane immunopositivity for E-cad in 40% of the cells present giving an ID score of 120 (× 400)

grading, grading by extent of necrosis, grading using Van Nuys, and by using the Cardiff classification.

Morphological classification of lesions was carried out using these four sets of criteria. Initially a classification based on nuclear morphology and polarity of the cells (architectural features) as described by Holland et al. [9] was carried out. The tumour cytology was assessed subjectively using the same criteria as applied in routine grading of infiltrating breast carcinoma irrespective of duct architecture or necrosis. Well-differentiated lesions showed uniform rounded regular nuclei with uniform chromatin staining and polarity of cells within the architecture of the malignant intraduct proliferation. Moderately differentiated lesions showed moderate nuclear pleomorphism with loss of polarity but some residual orientation of tumour cells. Poorly differentiated lesions showed marked nuclear pleomorphism, coarse chromatin and hyperchromasia with loss of polarity of cells. Nuclear morphology of DCIS was uniform within each individual case.

Next, a grading dependent on the extent of intraduct necrosis as described by Douglas-Jones et al. [4] was performed. This analysis was made on the basis of categorising the lesion/case as a

whole as either pure comedo (i.e. >90% of ducts containing necrosis), DCIS with necrosis (DN+) or DCIS without necrosis (i.e. less than 10% of ducts containing necrosis) (DN–).

The Van Nuys grading as described by Silverstein et al. [23] requires that lesions with high grade (grade 3) nuclear features with or without necrosis were placed in the high grade group. Non high grade DCIS was divided by the presence (group 2) or absence (group 1) of comedo type necrosis. Necrosis was defined as eosinophilic debris containing 5 or more pyknotic nuclei. Occasional desquamated or individually necrotic cells were ignored and were not scored as comedo type necrosis. No minimum extent of comedo type necrosis was required (one duct containing 5 or more pyknotic nuclei was sufficient to move a case from group 1 to group 2).

The Cardiff classification [8] uses a combination of scores for cytological grade and intraduct necrosis. Cytological grade is assessed as described for Holland. Low cytological grade=1 intermediate cytological grade=2, and high cytological grade=3. Intraduct necrosis is assessed as:

no necrosis (1), necrosis present in at least 1 duct lumen where necrosis is defined as the presence of 5 or more pyknotic nuclei (2) and where the duct lumen contains necrosis with more than 50% of any one duct diameter involved (3). These observations are combined to produce an overall score. Cases are considered to be well differentiated if they score 2 or 3, moderately differentiated if they score 4 or 5 and poorly differentiated with a score of 6.

For immunocytochemical staining of E-cad the indirect immunoperoxidase method was employed using the anti E-cadherin HECD-1 (human epithelial cadherin-1) mouse monoclonal antibody. The antibody has been previously characterised and its specificity described elsewhere [20]. Briefly, 5 μ sections were cut from blocks of formalin-fixed, paraffin-embedded tissues and dewaxed. After blocking endogenous peroxide, sections were subjected to autoclave pretreatment (120°C, 15psi, 10 min). Sections were cooled in running tap water and transferred to phosphate buffered saline (PBS) and immunostained. Immunostaining was done by incubating the tissue sections with 1 in 2 dilution of HECD-1 at 40°C overnight. The tissue binding of the antibody was disclosed using a standard peroxidase-antiperoxidase technique employing diaminobenzidine dihydrochloride (DAB) as the substrate and sections were counterstained with haematoxylin. Positive control tissue sections of normal small intestinal mucosa were used to ensure accurate and reproducible staining. Negative controls were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins. Immunostained sections were examined under a double-headed microscope by two observers and assessment was performed by con-

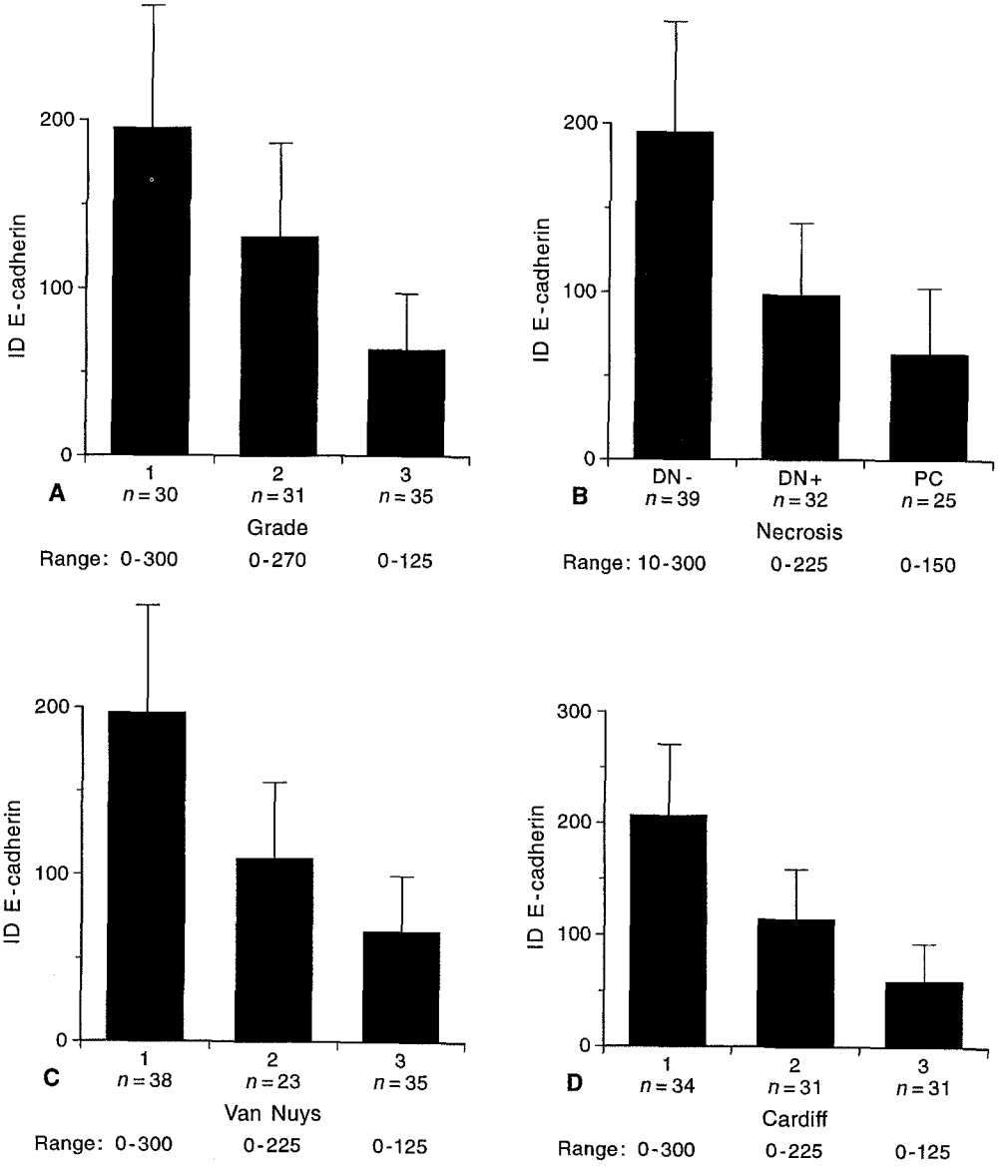


Fig. 4 The level of E-cad immuno staining (as measured by the ID scoring method) is plotted against the morphological differentiation of DCIS as assessed by various classification systems; **A** cytological differentiation (1=low, 2=intermediate, 3=high), **B** extent of necrosis (DN–=no necrosis–<10% of ducts show necrosis, DN+=with necrosis– 10–90% of ducts contain necrosis, PC=Pure Comedo >90% of ducts contain necrosis), **C** Van Nuys (1=non-high grade without necrosis, 2=non-high grade with necrosis, 3=high cytological grade) **D** Cardiff (1=well differentiated, 2=moderately differentiated, 3=poorly differentiated)

sensus. The amount of E-cad expression was assessed semi-quantitatively using an intensity distribution method. This method has been shown to be reproducible and to correlate with biochemical methods [12].

Initially the overall uniformity of positivity was assessed. If staining was uniform, three high power fields (field area=0.38 mm^2) containing DCIS within three different ducts were assessed as described below. The intensity distribution (ID) score for the case was the mean of these values. In the few cases where staining was not uniform, three of the most positive ducts were assessed and three of the weakest staining ducts present were assessed and the overall ID score was taken as the mean of these values. Within an individual ductal structure containing malignant epithelium, the total percentage of positive cells was assessed. Then the percentage of weakly, moderate and strongly staining cells was assessed, such that the sum of these categories equated with the overall percentage positivity. A staining score was then calculated as follows:

Score (out of maximum of 300) = sum of 1 × percentage of weak (+), 2 × percentage of moderate (++), 3 × percentage of strong (+++).

As the data was not normally distributed, a non-parametric multiple comparison test (Nemenyi-Dunn) was used to establish the statistical significance of the correlation between E-cad expression and the differentiation of DCIS as assessed by the four different classifications.

Results

The background "normal" breast epithelial cells showed uniform strong (+++) linear membrane staining for E-cad. Within the ducts containing DCIS, E-cad immunostaining was quite uniform with little variation in intensity either within individual ducts or between different ducts. In well-differentiated DCIS there was strong uniform membrane positivity (Fig. 1). In some cases of poorly differentiated DCIS this positivity was maintained (Fig. 2) but in other cases there was marked reduction in the E-cad expression (Fig. 3). In 5 cases there was total loss of detectable immunopositivity for E-cad (ID score=0) of which 1 was well-differentiated, 1 was moderately differentiated, and 3 were poorly differentiated. The expression of E-cad in DCIS of the breast is plotted against the cytological grade, extent of necrosis, Van Nuys classification and Cardiff classification in Fig. 4.

Table 1 (DN–, no duct necrosis; DN+, duct necrosis; PC, pure comedo)

Classification	Comparison	Q value	*p* value
Cytological Differentiation	1 v 2	2.74	<0.05
	1 v 3	6.94	<0.0001
	2 v 3	4.15	<0.001
Extent of necrosis	DN– v DN+	5.11	<0.001
	DN– v PC	6.85	<0.0001
	DN+ v PC	2.01	<0.1
Van Nuys	1 v 2	3.88	<0.001
	1 v 3	7.49	<0.0001
	2 v 3	2.72	<0.05
Cardiff	1 v 2	4.23	<0.001
	1 v 3	7.58	<0.0001
	2 v 3	3.24	<0.001

The results show significantly more expression of E-cad in grade 1 well-differentiated DCIS when compared with grade 2 (moderately differentiated) and grade 3 (poorly differentiated) DCIS. This trend is clearly seen and is significant irrespective of the grading system used (Table 1).

Discussion

E-cad is a protein expressed on the surface of epithelial cells which allows adhesion between adjacent cells. E-cad homophilic calcium-dependent interactions forming a cell adhesion "zipper" are thought to be the most important mechanism maintaining cell to cell contact in mammalian epithelium [10]. It is the only known non-junctional adhesion molecule in contrast to the gap junction desmosome and zonular adherens which are junctional adhesion mechanisms. There is a body of evidence supporting the hypothesis that normal mammalian epithelial cells express E-cad in order to maintain cell to cell contact. E-cad is localised on the cell membrane in areas of cell to cell contact [10]. Cells lacking E-cad expression do not aggregate in vitro and deletion of the E-cad gene locus [24], mutations in the E-cad gene [1] and low calcium in the intercellular environment lead to cell dissociation [27]. Introduction of E-cad c DNA into cells lacking a functional E-cad gene leads to E-cad expression and the ability of cells to aggregate [15].

Alpha catenin is a protein which links E-cad to the cytoskeleton of the cell. The presence of a functional catenin is also essential for cell aggregation and cells without catenin will not aggregate even if E-cad is expressed [10, 25]. This means that the demonstration of E-cad immunopositivity does not always imply the presence of functional adhesion. Results of E-cad studies must be interpreted with this in mind. It is known that the level of expression of E-cad is reduced in malignant cells [20, 21]. This is the probable basis for the loss of cell-to-cell cohesion in malignancy. Clearly the ability of cells to become detached from each other may be an important aspect of their ability to infiltrate stroma locally, their ability to penetrate endothelial cells and their ability to metastasise in lymphatics or vascular channels. Loss of E-cad appears to be a plausible explanation for the single cell infiltration patterns seen in lobular carcinoma of the breast and diffuse signet ring cell type of gastric carcinoma [1, 14].

DCIS of the breast is thought to represent the earliest stage of malignant transformation of cells of the terminal duct lobular unit which initially proliferate, filling and distending either lobular or ductal spaces but still bounded in the pre-invasive stage by the basement membrane of the ducts. At some point in the natural history of the disease (probably after many years) mutational events occur in the clonal expansion of intraductal cells which permit invasion through the basement membrane and surrounding stroma of the breast. In recent years it has become recognised that DCIS of the breast is not a ho-

mogeneous entity either morphologically or biologically [28]. At one end of the spectrum, the proliferating intraduct population is composed of cells with relatively regular rounded nuclei which may show polarity to a lumen forming solid, cribriform or papillary patterns and which are likely to express oestrogen receptor (ER), progesterone receptor (PR) and pS2 protein and be unlikely to express the oncogene product c-erb B2, the epidermal growth factor receptor (EGFR) and show little proliferative activity as assessed either by mitotic rate or by cell proliferation markers (MIB 1, Ki 67, S phase fraction in flow cytometry, PCNA expression or mitotic activity). At the other end of the spectrum ducts are distended by highly pleomorphic cells with large nuclei which show no polarity towards a lumen, are anarchic in their growth pattern and may or may not show central necrosis in ducts. This high grade DCIS shows less ER, PR, pS2 positivity and shows increased levels of c-erb B2, EGFR and p53 and shows proliferative activity [18]. These features have led to classifications of DCIS either on morphological or biological grounds and using a variety of criteria [5]. In this study we have used a classification based on cytology alone, a classification based on the extent of necrosis alone, a classification using elements of both (the Van Nuys classification) and a newly devised classification using a combined score of cytological appearances with extent of necrosis. Each of these classifications of DCIS has been shown to correlate with the grade of the invasive carinoma to which they give rise and with the prognostic index of the patient as a whole [5, 8]. The results presented here show that as would be expected E-cad expression is significantly higher in the most differentiated grade of DCIS and that E-cad expression is reduced in the least differentiated grade. These findings suggest that progressive loss of E-cad expression may occur at an early stage of breast carcinoma development. Progressive reduction of E-cad expression has been correlated with loss of differentiation and presence of metastatic disease in many human carcinomas including bladder, prostate, endometrium and breast [2, 19, 22, 25, 26]. In the colon it has been shown that there is reducing E-cad expression with progression of carcinogenesis. Hence, E-cad expression is maximal in normal mucosa and progressively reduced through adenoma, primary carcinoma and metastatic tumour [6]. This observation is in accord with the finding in this study that there is progressive loss of E-cad with loss of differentiation in DCIS (Fig. 4). In infiltrating breast carcinomas loss of expression of E-cad correlates with high histological grade, histological type, loss of ER and PR receptors and with axillary node metastasis [7, 14, 16, 17, 22]. However, not all studies have confirmed these findings [11]. Siitonen et al. studied E-cad expression in 362 infiltrating breast carcinomas using frozen sections and confirmed loss of E-cad expression associated with high histological grade, loss of oestrogen receptor, axillary node metastasis and shortened disease free survival in 109 patients in whom follow-up was available [22]. Within this study on frozen material there were 20 cases of pure DCIS of which 4 (20%) were reported as showing reduced or loss of E-cad expression. However, there was no further analysis of E-cad immunopositivity in DCIS in this paper. In our data 21 of 96 cases (22%) of pure DCIS showed weak or absent immuno staining (as evidenced by an ID score of less than 25% of maximum, i.e. an ID score of less than 75 out of 300). Of these lesions expressing weak or absent immuno staining 16 of 21 (76%) were poorly differentiated DCIS (all classifications). In our series, only 5 (5%) cases were completely negative for E-cad staining (ID score 0).

In summary this study has shown that there is reduced expression of E.-cad in pre-invasive breast carcinoma. This loss of expression has been related to the degree of differentiation of the DCIS as assessed by a variety of distinct morphological features. These findings reflect those found in pre-invasive colonic neoplasia and infiltrating carcinoma of the breast where reduced E-cad expression has been observed related to the grade of carcinoma as assessed by well-established grading methods.

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