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Loss of E-cadherin expression associated with lymph node metastases in small breast carcinomas

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Abstract The National Breast Screening Programme affords the opportunity to study breast carcinomas at an early stage in their development. E-cadherin is a calcium-dependent, intercellular adhesion molecule whose loss of expression may facilitate the processes of invasion and metastasis of some human tumours. From a group of screen-detected ductal carcinomas less than or equal to 10 mm in diameter, 16 with lymph node metstastasis were identified and matched for grade, size and patient age with node negative tumours. The level of expression of E-cadherin (detected by immunocytochemistry) was compared in the matched pairs using a simple semi-quantitative intensity distribution scoring system. The results showed a significant (P = 0.05 Wilcoxon paired rank test) reduction of E-cadherin expression in tumours with lymph node metastases compared to those without. In the context of the small size of these tumours it is proposed that these results support the hypothesis that reduction in E-cadherin expression is an early event in the development of metastases.

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

Introduction

There has been increasing interest in the structure and function of molecules that mediate cell-to-cell and cell-to-matrix adhesion in normal mammalian tissues and in malignancy. These molecules include the integrins, selectins, the immunoglobulin supergene family and the CD44 molecule. These families of molecules mediate adhesion between cells of the same type, between cells of different lineages or between cells and intercellular matrix [16]. The cadherin family of molecules is made up of transmembrane glycoproteins which mediate cell-to-cell adhesion via calcium-dependent, homotypic (cadherin-to-cadherin) interactions. Within this family there are molecules mediating adhesion between cells in different tissues. The most intensively studied is the E-cadherin (E-cad) molecule found on epithelial cells [20]. The E-cad molecule is linked to the cytoskeleton of the cell through alpha catenin. Loss of expression or function of any part of the E-cad–catenin–intracellular skeleton complex causes loss of calcium-dependent intercellular adhesion with loss of polarity, architecture and the ability to invade collagen gels [7, 22, 24].

In tumour biology, loss of intercellular adhesion between tumour cells is likely to be an important step in carcinogenesis, allowing stromal invasion, vascular invasion and metastasis. Decrease or loss of E-cad protein expression has been found in a wide range of tumours, including tumours of the cervix, endometrium, prostate, bladder and breast [2, 5, 8, 11, 15, 18, 23, 25]. It has been suggested that loss of E-cad may be related to metastatic potential in breast carcinoma [13, 21].

The aim of the present study was to define the role of loss of E-cad expression in the ability of early (small) breast carcinomas to metastasise to regional axillary lymph nodes.

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Materials and methods

Case material

We identified 213 otherwise unselected early (small, 10 mm or less) infiltrating duct carcinomas of breast that had been diagnosed in the course of the National Breast Screening Programme in South Wales. In this series, there were 24 (11.2%) tumours in which lymph node metastasis had already occurred. All cases were ductal carcinomas, NOS.

Table 1 Charactaristics of the cases of metastatic ductal carcinoma less than or equal to 10 mm diameter to which non-metastatic controls were matched

Grade	Size (mm)	Age (years)	Lymph nodes no positive/ no sampled
1	8	54	1/5
1	9	55	1/16
1	7	56	2/15
1	7	51	1/12
1	8	57	3/21
1	9	66	1/7
1	8	53	2/15
1	10	51	2/16
2	10	61	4/8
2	10	65	1/9
2	8	64	1/14
2	9	68	1/7
3	5	52	3/4
3	3	59	2/18
3	4	62	14/17
3	8	55	3/29

In some cases tumour blocks were not available, and in other cases where the tumour was very small there was no tumour tissue remaining after diagnostic assessment. There were 16 cases of metastatic ductal carcinoma NOS less than or equal to 10 mm diameter available for use in this study (Table 1). The size (in millimetres) as measured in the glass slide, grade as determined by Bloom and Richardson (Elston's modification [3]) and age of patient at diagnosis were known. From the pool of 189 remaining node negative, small (10 mm or less) carcinomas, control cases of ductal carcinoma, NOS were selected that matched the metastatic tumours for size (complete match except for one case with a 1-mm discrepancy), grade (complete match) and, as closely as possible, the age of patient (greatest mismatch 6 years). There were therefore two groups of size- and grade-matched ductal carcinomas, NOS of breast, all 10 mm or less in diameter, one group being node positive and the other node negative.

Immunocytochemical staining for E-cad

The indirect immunoperoxidase method was employed using the anti E-cad HECD-1 (human epithelial cadherin-1) mouse monoclonal antibody. The antibody has been characterised and its specificity described elsewhere [19]. Briefly, 5-μm sections were cut from blocks of formalin-fixed paraffin-embedded tissues and dewaxed. After blocking of endogenous peroxidase, sections were subjected to autoclave pretreatment (0.01 M sodium citrate buffer; pH 6.5; 120°C; 15 psi; 10 min). Sections were cooled in running tap water, transferred to phosphate-buffered saline (PBS) and immunostained. Immunostaining was done by incubating the tissue sections with 1 in 2 dilution of HECD-1 supernatant (20 μg/ml IgG 1) at 4°C overnight. The tissue binding of the antibody was disclosed using a standard peroxidase–antiperoxidase technique employing diaminobenzidine dihydrochloride as the substrate and sections were counterstained with haematoxylin. Positive control tissue sections which included normal appendix mucosa were used to ensure accurate and reproducible immunostaining (Fig. 1a). Negative controls were duplicate sections in which the primary antibody was omitted and replaced by normal mouse immunoglobulins.

The amount of E-cad expression was assessed semi-quantitatively using an intensity distribution (ID) method. This method has been shown to be reproducible and to correlate with biochemical methods [10].

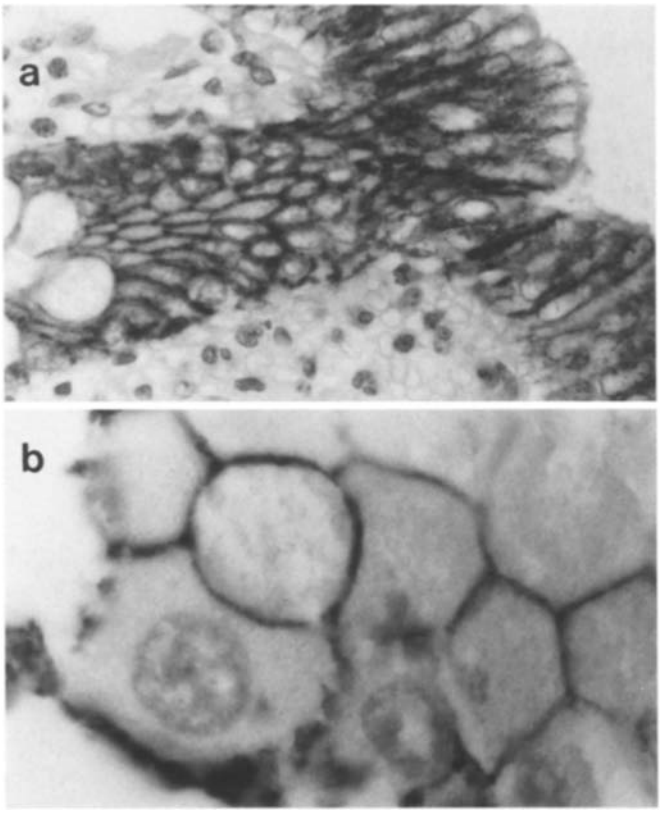

Fig. 1 **a** Paraffin embedded large bowel (appendix) columnar epithelium immunostained for E-cad as a positive control showing strong membrane positivity. ×600. **b** Background breast epithelium showing strong E-cad immunopositivity. ×1000

Analysis of staining: intensity distribution scoring of E-cad staining

Assessment of the immunopositivity was performed by two observers (N.C.A.H. and A.G.D.-J.) concurrently by consensus over a double-headed microscope. The sections were assessed without knowledge of any of the other parameters of the case (grade of tumour, age or lymph node status). Within a randomly selected high-power field (×63: field area = 0.061 mm^2) containing invasive tumour, the total percentage of positive cells was assessed. Then the percentage of weakly, moderately and strongly positive cells was estimated, such that the sum of these categories equated with the overall percentage positivity. An immunopositivity score was then calculated as follows:

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

For paired samples a Wilcoxon paired rank test was used.

Results

Columnar epithelium in normal appendix showed strong positive membrane immunopositivity (Fig. 1a), and the 'background' ductal epithelium of the breast showed delicate positive membrane staining for E-cad in all cases (Fig. 1b). No cytoplasmic positivity was seen in background epithelium. There was no positive membrane staining on myoepithelial cells. Some tumours showed

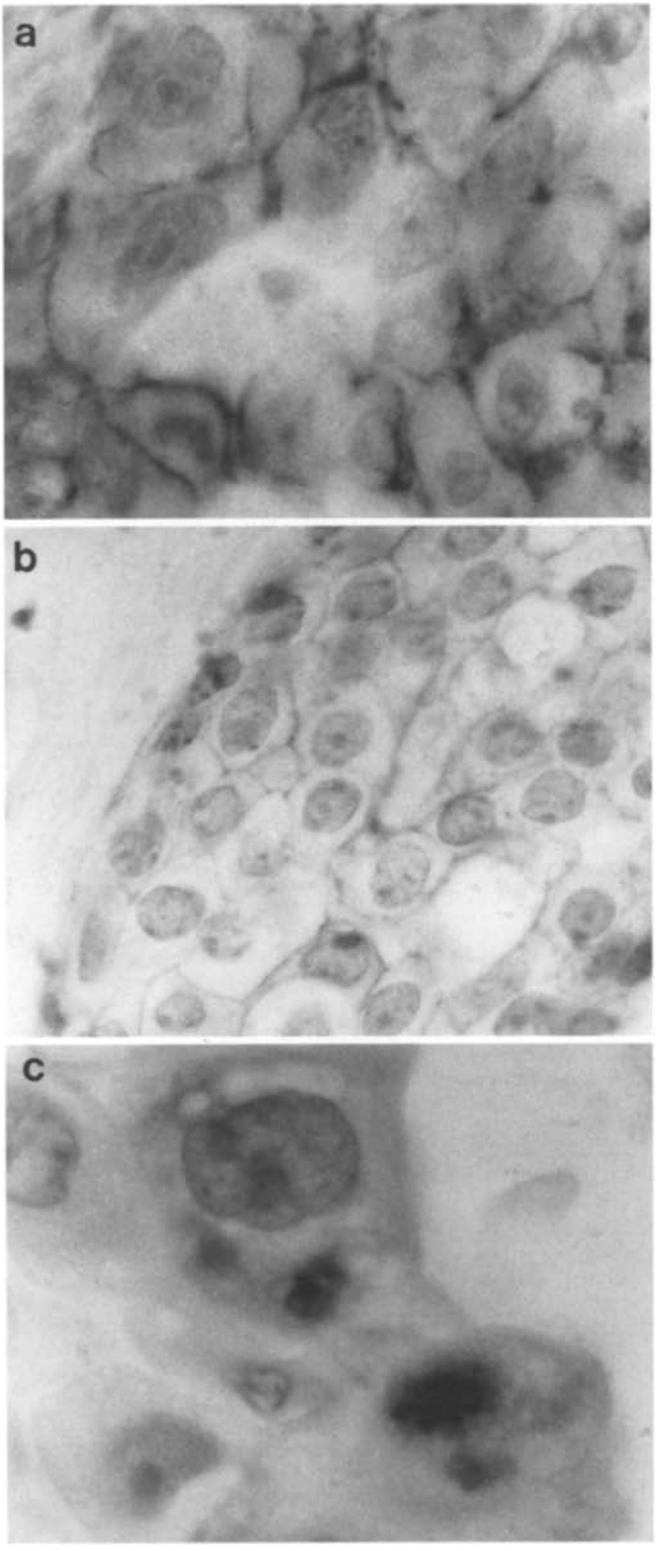

Fig. 2 **a** Infiltrating duct carcinoma of breast showing strong membrane immunopositivity for E-cad [intensity distribution (ID) score = 200)]. ×1000. **b** Infiltrating duct carcinoma of breast showing weak membrane immunopositivity for E-cad (ID score = 48) ×1000. **c** Dissociated tumour cells in a grade 3 carcinoma, showing cytoplasmic accumulation of immunopositive E-cad, which is thought to be non-functional ×1200

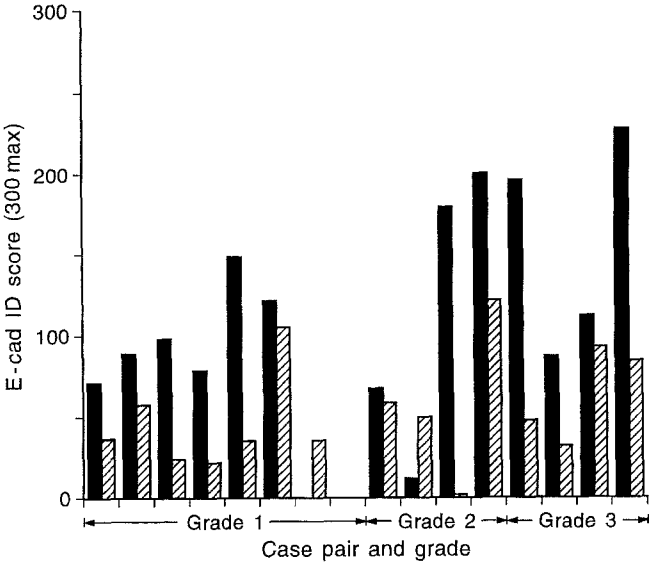

Fig. 3 ID scores of case pairs matched for size, grade and patient age (*hatched columns* with axillary lymph node metastasis, *solid columns* without axillary lymph node metastasis)

strong linear or densely dotted immunoreactivity of the cell membranes of E-cad where they were in contact with neighbouring cells (Fig. 2a), whereas other tumours were completely negative for E-cad expression and yet others showed faint but residual staining (Fig. 2b). The ID scores of tumours with and without metastases are shown in Fig. 3. The mean ID score for E-cad in the metastatic group was 49.4 (SD = 38) and the mean ID score of those tumours without metastases was 104.7 (SD = 70). The loss of E-cad immunopositivity in each of the tumour pairs (i.e. ID score of tumour without metastasis minus ID score of tumour with metastasis) was least for grade 1 tumours (mean loss = 44.8, $n = 7$) and greatest for grade 3 tumours (mean loss = 91.7, $n = 4$). In 5 cases there was apparent cytoplasmic accumulation of E-cad (Fig. 2c). Three of these tumours were grade 3; one was grade 2 and one grade 1.

There were 16 paired samples available for analysis. Statistical analysis was performed using a Wilcoxon paired-rank test and showed a highly significant difference between the E-cad immunopositivity of those tumours with lymph node metastases and those without ($P < 0.01$).

Discussion

There is in vitro evidence that expression of E-cad is important in the development of invasiveness. Blocking E-cad on canine kidney cell lines with antibodies confers an ability to invade collagen gels [1]. Transfection of the *E-cad* gene into colon carcinoma and fibroblast cell lines restores expression and reverses the malignant phenotype [9, 12]. Loss of E-cad expression has been documented in numerous human carcinomas, including those of cervix, endometrium, prostate, bladder, and breast [2, 5, 8,

11, 18, 23, 25]. Loss of E-cad expression has been associated with an infiltrative growth pattern in basal cell carcinoma of the skin [17] and associated with reduced survival in prostate and bladder carcinoma [2, 23]. In the breast, the characteristic single-cell infiltration pattern seen in lobular carcinoma has been shown to be associated with consistent loss of E-cad immunopositivity [11]. Oka et al. [13] showed that there was significant loss of E-cad positivity in breast tumours with metastatic disease. This study showed more E-cad loss with increasing size, but the size range in the tumours was large (10–50 mm). The quantitation of E-cad immunostaining was very crude, using two categories, either preserved or reduced. Of 22 patients with haematogenous or lymph node metastasis, 19 were evaluated as showing reduced or negative E-cad immunoreactivity [13]. In cervical intraepithelial neoplasia (CIN) increased cytoplasmic localisation of E-cad immunocytochemically correlated with the grade of the CIN lesion. In invasive squamous cell carcinoma of the cervix increasing loss of membrane and increasing cytoplasmic E-cad staining was seen with loss of differentiation [25]. The presence of intracytoplasmic E-cad is thought to imply non-functioning protein, which may be situated in the Golgi apparatus rather than in the cell membrane owing to abnormal processing of the precursor peptide, abnormalities in cytoskeleton or loss of alpha catenin. In 5 cases, we observed groups of cells in some areas showing intracytoplasmic positivity, and this finding was associated with rounding up of tumour cells and loss of cohesion (Fig. 2c). Similar "plaque-like" intracytoplasmic structures have been reported in undifferentiated gastric carcinoma [4].

In this paper we have examined the hypothesis that loss of E-cad expression by tumour cells has a role in the metastatic process of carcinoma of the breast to axillary lymph nodes. We have deliberately chosen small breast carcinomas which would be expected to be in a relatively early stage of their progression. There is an assumption that these tumours express the phenotype most likely to be important in invasion and metastasis before further irrelevant genetic events and phenotypic changes have occurred. Small tumours, 10 mm or less in diameter were rarely diagnosed in symptomatic practice prior to the advent of the National Breast Screening Programme. Size of primary tumour is known to be an important independent prognostic variable [14], and breast cancers 10 mm or less in diameter that have metatasised to axillary lymph nodes are even less common. In this study we have selected 16 of these unusual cases and matched them for other important prognostic parameters with 16 tumours drawn from a larger group of small ductal carcinomas that have not metastasised. We have shown that there is a significant difference in the E-cad expression as assessed semi-quantitatively between these two groups. In this series of small ductal carcinomas with metastases there were 7 grade 1 lesions and 4 each of grade 2 and grade 3, which reflects the known weighting towards grade 1 tumours in a screened population [6]. Although loss of E-cad expression is associated with metastatic disease, as demonstrated in this study and by others, the metastatic tumour in pair 12 retained an ID score of 121.7 (which is 53% of the maximum E-cad expression observed in any of the tumours), whereas the non-metastatic tumour in pair 10 had an ID score of only 10. These observations indicate that loss of E-cad expression is not the only, or even the most important, factor involved in the metastatic process. Moreover, the striking loss of E-cad in lobular carcinoma may explain the distinctive growth pattern but is not associated with excessively high rates of metastasis. There is a wide range of E-cad immunopositivity in both the non-metastatic (range = 0–226.7) and the metastatic (range = 0–121.7) tumours, and thus it is most unlikely that measurement of E-cad expression will be capable of accurate prognostication in an individual case.

This study has been designed to examine the role of E-cad loss in the metastatic process (as opposed to stromal invasion) in breast cancer. Small (early) carcinomas have been selected to reduce the significance of irrelevant genetic and phenotypic events. In matched pairs, there is consistently less E-cad immunopositivity in the metastatic tumour. Although it is certain that there are other important phenotypic alterations in the development of metastatic potential, this study indicates that loss of E-cad expression is one of them.

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