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Original Paper

Assay of E-cadherin by ELISA in Human Breast Cancers

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E-cadherin is a membrane-bound adhesion glycoprotein. Loss of E-cadherin has been correlated with invasion and metastasis in model systems. Using a new ELISA, we found higher levels of E-cadherin in fibroadenomas than in primary breast cancers. Levels in primary cancers showed no significant relationship with either tumour size, nodal status or oestrogen receptor levels. Patients with breast cancers containing low levels of the adhesion protein had a significantly shorter disease-free interval than patients with high levels ($P = 0.041$). The prognostic value of E-cadherin, for disease-free interval, was also found in node-negative patients as well as in patients presenting with small tumors (≤ 2 cm). In conclusion, loss of E-cadherin expression in human breast cancers is associated with increased metastatic potential as has previously been found in model systems. Loss of E-cadherin is thus likely to contribute to breast cancer progression.

Key words: E-cadherin, ELISA, breast cancer, prognosis

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INTRODUCTION

CADHERINS REPRESENT a superfamily of transmembrane glycoproteins characterised by a distinctive sequence motif which is tandemly repeated in their extracellular segments [1]. They function as calcium-dependent cell–cell adhesion receptors, playing a role during both embryonic development and in the maintenance of adult tissue architecture [2]. Several types of cadherins have been identified, including epithelial or E-cadherin (also called uvomorulin, L-CAM, cell-CAM 120/180), placental or P-cadherin and N-cadherin, which is found in muscle and adult neural tissue [3].

Of these different caherins, only E-cadherin has been widely investigated in malignancy. Several studies have now shown a relationship between decreased expression of this adhesion protein and increased propensity of malignant cells to invade and metastasise (for review, see [4]) Firstly, a negative correlation exists between the expression of E-cadherin and invasive potential for many different cancer cell lines. Secondly, in cell lines lacking E-cadherin, invasion was prevented by transfection with cDNA for this cadherin. Thirdly, reduction in E-cadherin mRNA levels by antisense sequences induced the invasive phenotype in E-cadherin-positive cells. Fourthly, antibodies inactivating E-cadherin induced the invasive phenotype. These experiments taken together are strong evidence that loss of E-cadherin is associated with development of an invasive phenotype, and furthermore suggests that this adhesion molecule may be a suppressor of metastasis.

The aim of this investigation was to study E-cadherin levels in human breast cancers using a new ELISA and to relate these levels to both established prognostic markers for this disease and patient outcome.

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MATERIALS AND METHODS

Tumours and patients

Following histological examination, breast tumours were snap-frozen in liquid nitrogen and then stored at −70°C until further use. Samples from patients with benign breast disease (i.e. fibroadenomas) served as controls. The main characteristics of the 150 primary carcinomas investigated are summarised in Table 1. The median follow-up period was 48.9 months. Both median values (267 ng/mg protein) and optimum cut-off points (65 ng/mg protein) were used when relating E-cadherin levels to patient outcome.

Table 1. Tumour size, nodal status and ER status of primary cancers used

	Primary breast lesions	
	n	(%)
Size		
⩽2 cm	43	29
>2 cm	68	45
Unknown	39	26
Nodal status		
Negative	58	39
Positive	50	33
Unknown	42	28
ER status		
Negative	60	40
Positive	74	49
Unknown	16	11

Extraction procedure

Tumours were homogenised in 50 mM Tris–HCl (pH 7.4) and centrifuged at 2000*g* for 10 min. Initially, a number of different detergents were evaluated for extracting E-cadherin. These included the non-ionic detergents Digitonin (20 μg/ml), *n*-dodecylmaltoside (10 mM), Nonidet P40 (1%), Triton X-100 (1%), Tween 20 (1%) and Brij 35 (1%) as well as the novel zwitterionic detergent CHAPS (10 mM). All detergents used were part of a detergent trial kit obtained from Boehringer Mannheim (Mannheim, Germany, Cat. No. 1652 826). As Tween 20 was found to give the highest yield, this was used in all subsequent extractions. The extraction involved incubating the tumour extract and detergent together for 1 h, shaking on ice. Following centrifugation at 10 000*g* for 15 min at 4°C, the supernatants were collected and used for the E-cadherin ELISA assays.

E-cadherin ELISA assay

E-cadherin antigen levels were detected in the supernatants using a human E-cadherin ELISA kit (Code no. MK017, Takara Schuzo Co., Ltd., Biomedical Group, Japan). This is a sandwich ELISA that utilises two mouse monoclonal antibodies to detect E-cadherin in a two-step procedure. One monoclonal antibody, designated HECD-1, recognises a site close to the N-terminus in the extracellular domain while the other antibody (SHE13-6) reacts with a region that seems to be just on the extracellular side of the transmembrane region.

Other assays

Tumours were analysed for the presence of oestrogen receptors by ELISA as previously described [5, 6]. The cut-off point was 200 fmol/g wet weight of tissue. Protein levels were assayed using the BCA Protein Assay Reagent manufactured by Pierce (Rockford, Illinois, U.S.A.) due to its compatability with both ionic and non-ionic detergents.

Statistics

All data was analysed by non-parametric methods using the Spearman rank coefficient or the Mann–Whitney U-test. Differences in E-cadherin levels between patient subgroups were determined by the chi-square (χ^2) test. Analysis of disease-free interval and survival were performed by the Kaplan–Meier method and tests for differences were made using the log rank method.

Table 2. Effect of different detergents on solubilising E-cadherin (where " + " sign denotes increased yield). For this experiment, extracts from five different tumours were pooled

Detergent	Final concentration	E-cadherin (ng/mg)	% change in yield
Tris buffer	50 mM	960	0
Digitonin	20 μg/ml	1700	+77
n-Dodecylmaltoside	10 mM	920	−4
Nonidet P40	1%	840	−12.5
Triton X-100	1%	968	+0.8
Tween 20	1%	2040	+112
CHAPS	10 mM	920	−4
Brij 35	1%	1320	+37.5

RESULTS

Extraction of tumour cytosols with detergents

As E-cadherin is a membrane-bound protein, initial experiments compared the ability of different detergents to extract this molecule. As shown in Table 2, levels of E-cadherin were found to be essentially unaltered by Triton X-100 (+0.8%), *n*-dodecylmaltoside (−4%), CHAPS (−4%) and Nonidet P40 (−12.5%) when compared to extraction with 50 mM Tris buffer alone. The only detergents which increased the yield were Brij 35 (+37.5%), Digitonin (+77%) and Tween 20 (+112%). As 1% Tween 20 was the detergent which gave the maximal yield of E-cadherin, this was used for all further extractions.

Levels of E-cadherin in malignant and benign breast tumours

Table 3 shows the median and range of E-cadherin in benign breast tumours (fibroadenomas), primary carcinomas and axillary node metastases. Levels were found to be decreased in primary carcinomas when compared to those with benign disease (Mann–Whitney U-test, $P < 0.05$). There was no significant difference between levels in the primary and metastatic carcinomas ($P = 0.639$) or between levels in benign and metastatic samples ($P = 0.215$).

Relationship between E-cadherin and established prognostic markers

E-cadherin levels were compared with the established breast cancer prognostic factors such as tumour size, axillary node status and oestrogen receptor levels. No significant relationship was found between E-cadherin levels and any of these parameters.

Table 3. Distribution of E-cadherin levels in primary and metastatic breast carcinomas compared to those with benign breast disease

Category	*n*	E-cadherin (ng/mg protein) Median	Range
Benign	10	467	166–5000
Primary carcinoma	150	267	0–4352
Metastatic carcinoma	13	190	40–2180

Relationship between E-cadherin levels and patient prognosis

Using the median level as the cut-off point, no significant association was found between E-cadherin levels and patient outcome. However, using an optimum cut-off point, i.e. 65 ng/mg protein, patients with low levels of the adhesion protein had a significantly shorter disease-free interval than those with high levels ($\chi^2 = 3.96$, $P = 0.041$, relative risk = 1.94; Figure 1a). Patients with low levels of E-cadherin also tended to have a shorter overall survival, but this relationship was not statistically significant ($\chi^2 = 1.52$, $P = 0.338$, relative risk = 1.46; Figure 1b).

E-cadherin was also investigated for potential prognostic value in different subgroups of patients with breast cancers. As shown in Figures 2 and 3, E-cadherin levels correlated with shorter disease-free interval in both node-negative ($\chi^2 = 4.24$, $P = 0.04$) and patients presenting with small tumours ($\leqslant 2$ cm; $\chi^2 = 4.83$, $P = 0.028$). E-cadherin, however, was not related to disease-free interval in node-positive patients or in patients with large tumours (>2 cm). Finally, E-cadherin was not significantly associated with overall survival in any of these subgroups.

DISCUSSION

This is one of the first reports to describe a quantitative assay to detect E-cadherin in a human cancer. Most previous reports for measuring this adhesion molecule have used immunohistochemistry [7–11], a semiquantitative procedure. Using ELISA, we showed no significant relationship between E-cadherin levels and established prognostic markers in breast cancer, such as tumour size, nodal status and

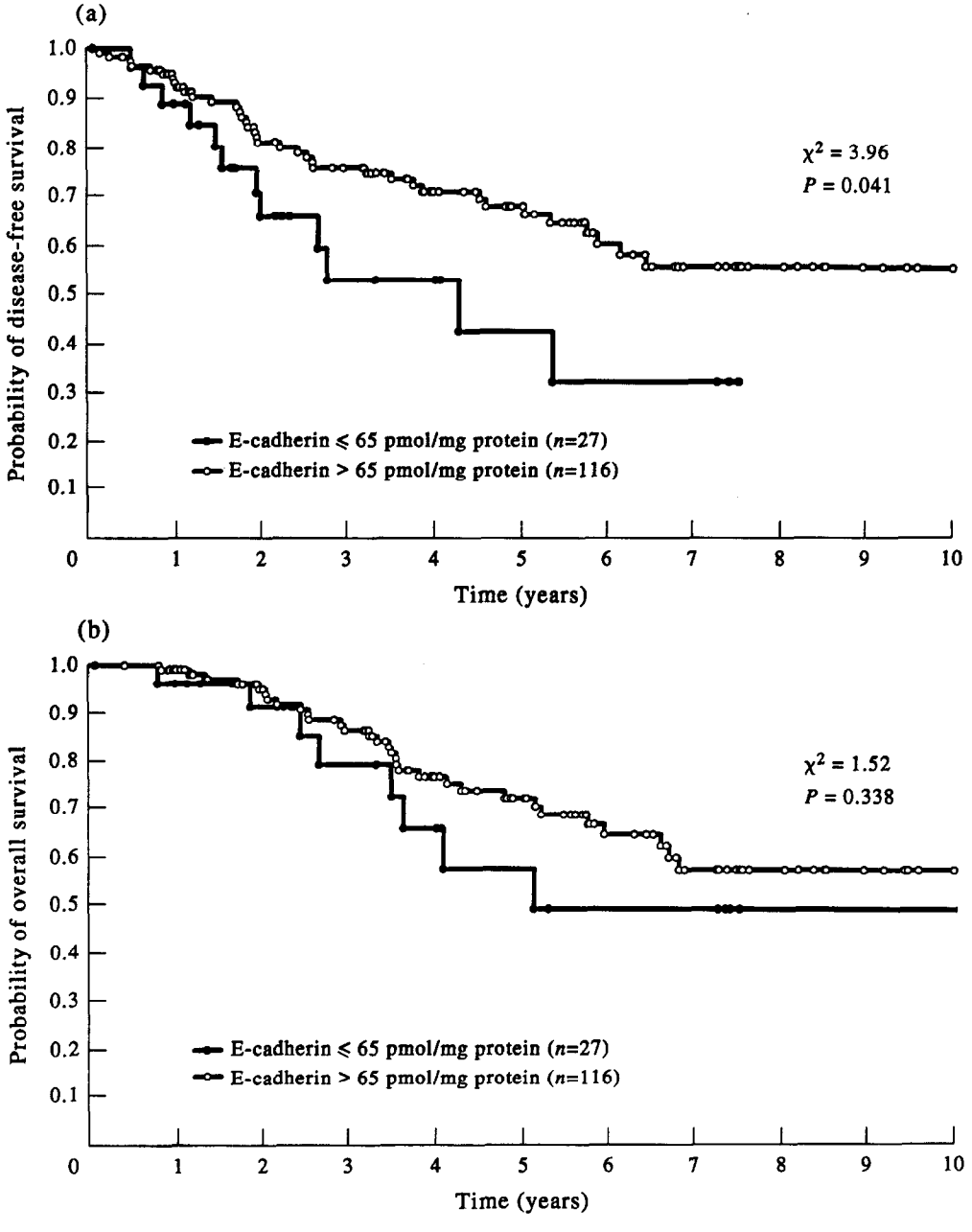

Figure 1. Relationship between E-cadherin levels and (a) disease-free interval and (b) overall survival in patients with breast cancer. Cut-off point for E-cadherin was 65 ng/mg protein.

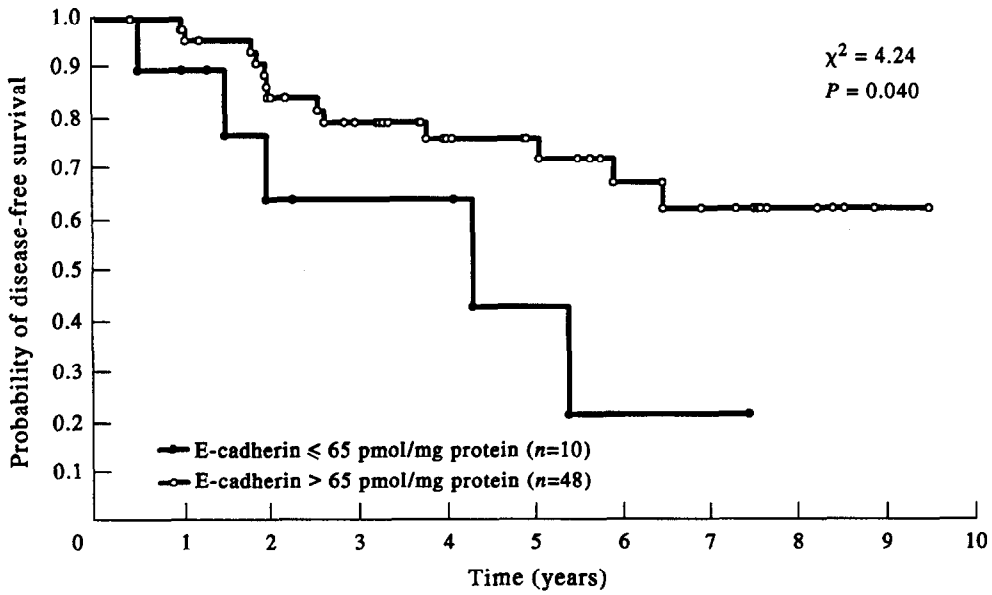

Figure 2. Relationship between E-cadherin levels and disease-free interval in node-negative patients with breast cancer. Cut-off point was 65 ng/mg protein.

ER status. Similar results have been obtained by others using immunohistochemistry to detect E-cadherin [12]. However, Oka and associates [13], also using immunohistochemistry, found a significant relationship between reduced type E-cadherin levels and both tumour size and nodal metastasis.

As mentioned in the Introduction, results from model systems show a relationship between loss of E-cadherin and potential for invasion and metastasis. Our results with human breast cancer are consistent with these findings. Here, we show that patients with low levels of E-cadherin in their primary tumours have a significantly shorter disease-free interval than patients with high levels. The prognostic impact of E-cadherin was strongest in those patients likely to have a good outcome, i.e. node-negative patients and patients presenting with tumours of diameter 2 cm or less. Clearly, these preliminary results need confirmation with larger numbers of patients and longer follow-up. To our knowledge, this is the first demonstration of a relationship between low levels of this cadherin protein and poor prognosis in breast cancer. However, low levels of E-cadherin protein or mRNA have been associated with aggressive disease in other malignancies such as prostate [14], renal [15], gastric [11] and colorectal [8] cancers. Thus, like certain proteases implicated in cancer spread [16], E-cadherin may be a prognostic marker for many different types of adenocarcinoma.

In conclusion, E-cadherin levels were reduced in malignant breast tumours relative to benign tumours. In the pri-

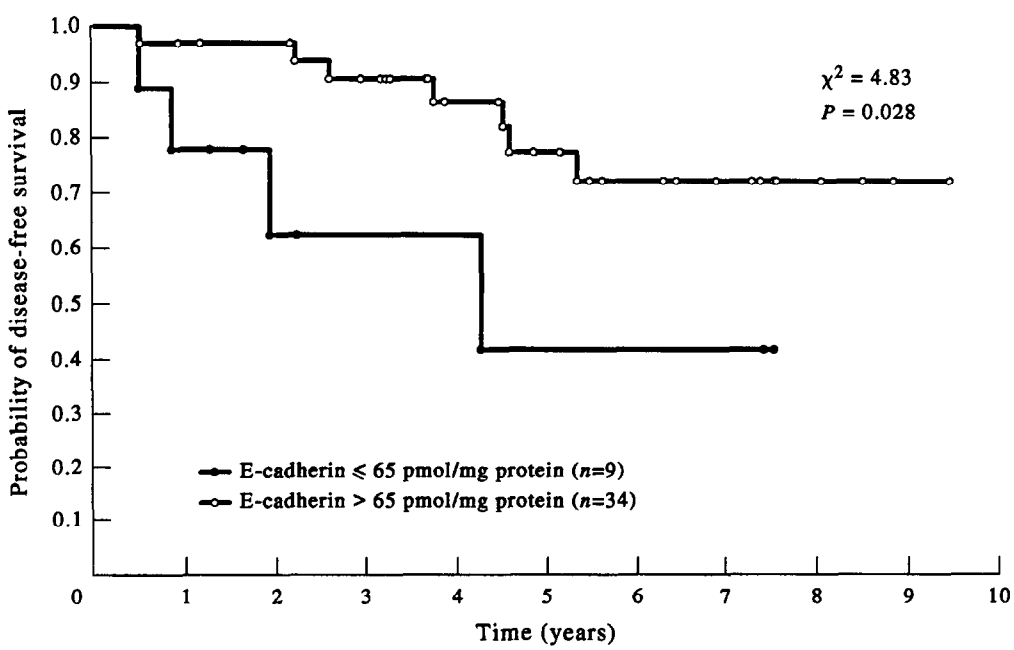

Figure 3. Relationship between E-cadherin levels and disease-free interval in patients with breast cancer with tumours ≤ 2 cm. Cut-off point was 65 ng/mg protein.

mary carcinomas, lower levels were significantly associated with shorter disease-free interval. Future work should be directed at confirming our preliminary results on the prognostic value of E-cadherin in patients with node-negative and small breast cancers, how reduced E-cadherin levels contributes to cancer spread and whether increasing the E-cadherin levels in malignant tissue might prevent metastasis. Of potential interest was the recent finding that tamoxifen restores the function of E-cadherin in a breast cancer cell line and at the same time suppresses the invasive phenotype [17]. Whether these *in vitro* actions of tamoxifen are relevant to its *in vivo* actions remains to be shown.

Note added in proof: While this manuscript was being evaluated, Siitonen SM, Kononen JT, Helin HJ, Rantala IS, Holli KA, Isola JJ in *American Journal of Clinical Pathology* 1996, **105**, 394–402, reported that reduced E-cadherin expression, as determined by immunohistochemistry, was also an unfavourable prognostic indicator in breast cancer.

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