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Cathepsin D Levels in Primary Breast Cancers: Relationship with Epidermal Growth Factor Receptor, Oestrogen Receptor and Axillary Nodal Status

Nigel P.M. Sacks, Kenneth Smith, Andrew P. Norman, Michael Greenall, Susan LeJeune and Adrian L. Harris

Cathepsin D and the epidermal growth factor receptor (EGFr) have both been proposed as poor prognostic markers in breast cancer. We have compared the tumour cytosolic cathepsin D level with EGFr and oestrogen receptor (ER) levels and the axillary node status of 131 patients with operable breast cancer, to see if EGFr and cathepsin D are co-regulated. Cathepsin D level was measured using a two-site immunoradiometric assay kit. No correlation was found between the level of cathepsin D and EGFr, ER or nodal status. Since the raised level of cytosolic cathepsin D was not related to EGFr, it may be that measuring the level of both of these markers in the same sample will give additional prognostic information.
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INTRODUCTION

THE IDENTIFICATION of more accurate predictors of relapse and survival in patients with primary breast cancer may help to select those who could benefit from postoperative adjuvant systemic therapy [1]. Whilst axillary lymph node involvement is currently the single best predictor of recurrence [2], approximately 30% of node-negative patients experience relapse within 5 years of the initial treatment [3].

Recently, several new prognostic factors have been proposed such as epidermal growth factor receptor (EGFr) overexpression [4, 5], amplification and/or overexpression of oncogenes such as HER-2/*neu* and c-*myc* [6, 7], and the measurement of the tumour proliferative activity using the thymidine-labelling index [8] or DNA flow cytometry [9].

Cathepsin D is an acidic lysosomal aspartyl endopeptidase with a molecular weight of approximately 42 kD which is synthesised as a 52 kD glycoprotein precursor [10]. Cathepsin D has been shown to be an independent predictor of prognosis in patients with primary breast cancer, particularly those with node negative disease [11–13]. The level of cathepsin D in breast cancer cytosol has been shown not to correlate with tumour grade, size, lymph node status, ER (oestrogen receptor) and PR (progesterone receptor) content [14], c-*erb*B-2, *int*-2 level in the tumour [11–14]. The mechanisms responsible for poor outcome of EGFr expressing tumours remain unknown, but could be due to growth factor induced protease production. We therefore assayed EGFr and cathepsin D coexpression. If independently expressed then further studies would be indicated to assess their use as a combined assay in predicting patient outcome.

Correspondence to A.L. Harris.
A.L. Harris, K. Smith, M. Greenall and S. LeJeune are at the ICRF Molecular Oncology Laboratories, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU; and N.P.M. Sacks and A.P. Norman are at the Royal Marsden Hospital, Fulham Road, London SW3 6JJ.
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MATERIALS AND METHODS

The study included 131 primary invasive ductal breast carcinomas. Axillary node sampling was performed in 123 cases. All surgical biopsy specimens were snap-frozen within 2 h of surgery and stored at −196°C until processing.

Tissue preparation

All procedures were carried out at −4°C. Tumours were pulverised in liquid nitrogen and further homogenised in a ratio of 1:20 (w/v) in buffer (0.02 mol/l Hepes, 0.00075 mol/l EDTA, 0.001 mol/l benzamidine, 0.0005 mol/l phenylmethylsulphonylfluoride, 1 mg/μl oromucoid, pH 7.4 at 20°C) using three 1 s bursts of a Polytron homogeniser (setting 6). The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was centrifuged at 37 000 rpm for 40 min. The pellet (crude membrane fraction) was resuspended in buffer and stored at −80°C until assayed for EGFr, the supernatant (cytosol) was made 0.002 mol/l with respect to dithiothreitol and separate aliquots were assayed for cathepsin D and oestrogen receptor. Protein concentrations were assayed using the method of Bradford with bovine serum albumin as standard.

Table 1. Relationship of cathepsin D expression to node status, ER and EGFr

	No. of cases	% Cathepsin D + (*n*)		% node + (*n*)
ER +	70	30	(21)	46 (30/65)
−	61	41	(25)	48 (28/58)
EGFr +	47	36	(17)	52 (24/46)
−	84	35	(29)	44 (34/77)
Cathepsin D +	46	35	(46)	52 (24/46)
−	85			44 (34/77)
Nodes +	58	41	(24)	47 (58/123)
−	65	34	(22)	

Nodes known in 123/131 cases.

Cathepsin D radioimmunoassay

Cytosols, prepared as described above, were stored at −80°C prior to measurement of cathepsin D. A one-step double determinant solid-phase immunometric assay previously described [14] was adapted for routine analysis (ELSA-cath-D kit) by CIS Bio-Industries (Gif-sur-Yvette, France). The ELSA solid phase is a multifinned stick coated with the antibody and jammed into the bottom of a tube. The assay involves two monoclonal antibodies, one (D7E3) coated on the ELSA solid phase and the other (M1G8) radiolabelled with ^{125}I. The assay was performed on diluted cytosols of breast cancer (three dilutions per sample: 1:20, 1:40, 1:80, and additional dilutions if necessary). In each ELSA tube, 300 µl of tracer and 50 µl of each standard or sample dilution were incubated for 3 h at room temperature with horizontal shaking. The tubes were then washed and counted in a Beckman gamma 5500B spectrometer with a counting efficiency of 76%. Cathepsin D concentration is expressed as pmol/mg cytosol protein.

ER assay

The ER content of the tumours was determined using the dextran-coated charcoal method and a multipoint radio ligand binding assay. Tumour specimens were considered ER positive if they contained at least 5 fmol of specific binding sites per milligram of cytosolic protein.

EGF receptor assay

EGF was iodinated by the Iodogen method and the EGFr in the tumours was determined as previously described [15] with a single point assay. A cut-off value of greater than 20 fmol/mg protein was used to differentiate between receptor positive and negative tumours.

Statistical methods

The differences in the medians of continuous variables was examined using the Mann–Whitney non-parametric test. The χ^2 test was used to examine differences in the discrete variables.

RESULTS

47% of patients were node positive and 53% ER positive. Cathepsin D concentrations and distribution were similar to those previously described with this assay [14]. (Mean 57 pmol/mg, interquartile range 29–75.2, range 11.7–326.3.) A cut-off value of greater than 60 pmol/mg was chosen as positive, based on the manufacturer's recommendations and previous data [14].

When tumours were categorised as ER positive or negative. EGFr positive or negative and cathepsin D positive or negative, there was no association of percentage of cases positive for cathepsin D with the other markers (χ^2) (Table 1). Node status also did not correlate with cathepsin D status (Table 1). Since cathepsin D is a continuous variable, the values were analysed by the Mann–Whitney test versus node status, ER and EGFr status (Fig. 1). There was no significant association.

DISCUSSION

This study shows that cathepsin D concentration in the primary breast tumour is independent of both nodal status and overexpression of the EGFr. We also found that the median level of cathepsin D was similar in the patients with involved nodes when compared with those with uninvolved axillary nodes, as have other workers [11, 14]. In contrast to one previous study [11] the level of cathepsin D was found to be unrelated to oestrogen receptor status. These disparate results may be partly due to the different cut-off levels of cathepsin D employed in each study; we used a cut-off level of 60 pmol/mg protein as recommended by the manufacturers of the assay kit (Gif-sur-Yvette, France) and Rochefort *et al.* [10]. This work also shows that cytosol prepared for ER and EGFr assays can be conveniently used for the total cathepsin D two-site immunoassay. It will therefore be important to assess the combined measurement of cathepsin D and EGFr in the prognosis of node negative breast cancer.

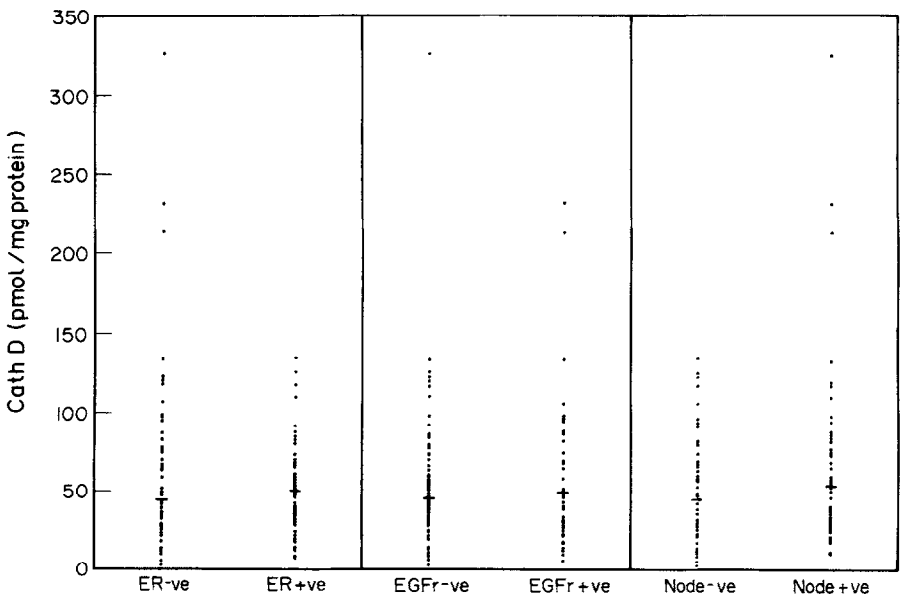

Fig. 1. Relationship between quantitative cathepsin D expression, ER, EGFr and node status.

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Feature Articles

U.K. DCIS Trial—Time for Review?

Hazel Thornton

INTRODUCTION

THE REPORT by van Dongen *et al.* [1] summarises the findings of the 55 participants who met to reach agreement about what is currently known about ductal carcinoma *in situ* (DCIS) with reasonable medical certainty and to define what questions remain unanswered.

DCIS has now been shown to have remarkable radiological, histological and biological diversity and the uncertain clinical implications of this heterogeneity indicate a tremendous need for continued research to determine patient management after breast-conserving surgery, particularly as many more cases are being found as a result of the breast screening programme. This heterogeneity, and indeed the problem of accurate diagnosis, is not a problem when mastectomy (which is almost 100% successful) is employed.

Disturbingly, many countries reported a reluctance of both physicians and patients to accept entry into these trials, but due to the heterogeneity of DCIS, ever larger numbers of participants will be required to achieve statistical power in the resultant subclassifications. It is, therefore, vital to determine causes for this reluctance by consideration of the patient's viewpoint [2] and an examination of the U.K. DCIS Trial which is precipitate, flouts World Health Organization (WHO) princples, has very unequal treatment options and wide participant entry.

THE UNACCEPTABLE ASPECTS OF THE PROTOCOL [3]

van Dongen reports: "The main endpoint of studies of breast conserving therapy (BCT) in DCIS should be prevention of deaths from invasive breast cancer, and not just progression to invasion or recurrent DCIS", and states that "much longer follow-up is needed...especially as radiotherapy might be associated with a delay in recurrence, and the median time to recurrence is very long anyway." The current U.K. DCIS trial does not have survival as its endpoint and the trial protocol itself acknowledges that, "Radiotherapy might be considered as over-treatment", and forsees the possibility of an unbalanced entry at 2 years with resultant closure of one arm of the trial.

It is surely questionable that radiotherapy is an appropriate treatment for a non-invasive carcinoma that may not progress to an invasive phase during the patient's lifetime, particularly as it

H. Thornton is at "Saionara", 31 Regent Street, Rowhedge, Colchester CO5 7EA, U.K.
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