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Immunostaining of Cathepsin D in Breast Cancer: Quantification by Computerised Image Analysis and Correlation with Cytosolic Assay

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Cathepsin-D (cath-D) was quantified in 34 breast cancer specimens by immunohistochemical staining of frozen sections with a computer image analysis and the results were compared with the corresponding cytosolic assay. Cath-D concentrations varied from 0 to 420 arbitrary units (AU). Tumour cells were more intensely stained than peritumoral tissue with the D7E3 mouse monoclonal antibody than with rabbit polyclonal antibodies. There was a good correlation ($r = 0.80$) between cath-D values obtained either by immunohistochemistry with D7E3 antibody or by cytosolic immunoenzymatic assay. However, with a cut-off of 50 AU, 3 out of 25 patients had higher immunohistochemical values and 2 had higher cytosolic values. Therefore, quantification of cath-D concentration in tissue section by immunostaining and a computerised image analyser, which is the only technique available for small tumours, should provide similar prognostic information to that obtained by assaying cath-D in the cytosol.
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INTRODUCTION

SEVERAL RETROSPECTIVE studies have shown that high cystosolic cathepsin D (cath-D) concentration in primary breast cancer is correlated with a higher frequency of relapse and metastasis [1, 2, 3, 4]. Moreover, during the progression from normal to malignant mammary cells, cath-D level is markedly increased [4, 5]. However, the pronostic value of cath-D was obtained from immunoassays performed in cytosol [1, 2] or total cell extracts [3] which require a large amount of tissue. Moreover, this assay does not discriminate between antigen produced by cancer cells and that produced by adjacent cells located in the connective tissue or vessels. Due to the increasing progress in early detection of breast cancer, prognostic markers should be quantified in small tumours. Immunohistochemistry in combination with computer-assisted image analysis might be able to fulfil this objective if its validity compared to the cytosol assay can be demonstrated. A good correlation between these two techniques was previously obtained with oestrogen and progesterone receptors [6, 7].

The first immunohistochemical study on cath-D in breast cancer with polyclonal rabbit antibodies to normal cath-D

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indicated that high cath-D levels in tumoral cells were correlated with good prognosis and the presence of oestrogen receptors [8], which contrasted with studies performed on cytosol.

The major aim of the present study was to standardise quantification of the immunohistochemical cath-D assay using D7E3 mouse IgG1 monoclonal antibody and to correlate it with the cytosolic assay. Secondly, we wanted to compare immunostaining of the same mammary tumour, obtained with either the mouse D7E3 monoclonal antibody or rabbit polyclonal antibodies described by Henry *et al.* [8], in an attempt to explain the contrasting results obtained by these authors concerning the prognostic significance of cath-D.

MATERIALS AND METHODS

Patient population

42 breast tumoral tissues were collected in liquid nitrogen and sent to the Department of Cell Biology of the University of Montpellier for biochemical receptor analysis.

Samples of greater than 1 cm diameter were required to perform both biochemical and immunohistochemical analyses. 85% of the population was T_2 to T_4 according to the TNM classification. All tissues were examined histologically by a pathologist for diagnosis and stored at −70°C. Frozen tissues were divided into two parts, one for cytosolic assay and the other for immunohistochemical staining.

Cytosol assays

Frozen tissues were homogenised in 10 mmol/l Tris (pH 7.4) containing 1.5 mmol/l ethylene diamine tetracetic acid (EDTA), 10 mmol/l monothioglycerol, and 10 mmol/l sodium molybdate (TET molybdate buffer) and centrifuged at 105 000 *g* for 60 min. Aliquots of the same high-speed supernatant (cytosol) were used for measuring concentrations of total cath-D, oestrogen receptors (ER) and progesterone receptors (PR). ER and PR were assayed using the Abbott enzyme immunoassay (ER-EIA and PR-EIA) [9]. 10 fmoles/mg protein were taken as being the limit of positivity for ER and PR. Protein concentration was assayed using a Bio-Rad kit (Lab GmBH, Munich, Germany) using bovine serum albumin (BSA) as the standard. Total cath-D was measured in a one-step double determinant solid-phase immunoenzymatic assay (IEMA).

Briefly, 100 µl of sample, were incubated for 3 h in ELSA-cath-D tubes (CIS Biointernational, Gif sur Yvette, France) with 250 µl of monoclonal antibody MIG8 labelled with alkaline phosphatase in a phosphate buffer (pH 7.4) at room temperature with horizontal stirring. Tubes were then washed three times with water containing 0.3% Tween 20. 350 µl of a paranitrophenyl phosphate (PNPP) substrate solution were added and incubated for 30 min at room temperature. The enzymatic reaction was stopped by adding 1 ml of NaOH (1 mol/l). Optical density was read in a computerised spectrophotometer (SP 500, Pasteur). A high correlation was obtained betwen this IEMA assay and the commercially available immunoradiometric assay ($r = 0.946$, $n = 40$) as previously shown between IRMA and immunoenzymatic assay on microplates [10].

The total cytosolic cath-D was measured in 421 cytosols from breast cancer patients collected from March 1989 to February 1991. The mean concentration (SD) was 73.4 (82) pmol/mg protein, whereas the mean value currently used as a cut-off level for prognostic use in several clinical studies is 50 pmol/mg protein.

Immunohistochemical staining of cathepsin D

Two different antibodies were used. First, D7E3 mouse monoclonal antibody raised against secreted pro-cath-D purified from breast cancer MCF7 cells [11] recognises three forms of cath-D (precursor, intermediate and mature). This monoclonal antibody is used to retain the antigen on the solid phase in the total cath-D IEMA. Secondly, rabbit polyclonal antibodies raised against the mature form of cath-D (28 000 + 14 000) from normal human spleen were kindly provided by Dr W.A. Reid (Department of Pathology, University of Leeds) [12].

In both cases, 5 µm thick frozen sections of breast cancer tissues were prepared on a cryocut (SLEE, London). Two serial sections were collected on a gelatin-coated glass slide for specific staining and control staining using the same class of antibody. They were immediately fixed for 10 min in 3.7% paraformaldehyde in 0.01 mol/l phosphate buffered saline (PBS), pH 7.3, 4 min in methanol at −20°C and 2 min in acetone at −20°C, then washed for 10 min in PBS.

The staining was performed using D7E3 monoclonal antibody as described [13] with slight modifications. Tissue sections were incubated for 60 min at room temperature with 100 µl of a 1 µg/ml D7E3 solution. The monoclonal antibody was then revealed with biotinylated antimouse antibody and avidin-peroxidase (ABC kit, Vectastin Burlingame, USA) using 3,3'-diamino benzidine tetrahydrochloride as substrate. For quantification, slides were counterstained with Harris haematoxylin, dehydrated and mounted.

Staining with rabbit antiserum was performed on frozen sections and not on paraffin embedded sections as originally described by Henry *et al.* [8]. Frozen sections were not pretreated with 0.1% trypsin and were incubated for 60 min with 100µl of a 1/300 dilution of the rabbit antiserum. The complex was then stained using antirabbit biotinylated species-specific whole antibody (from donkey) and avidin-peroxidase (ABC kit) instead of peroxidase–antiperoxidase stain.

Specificity of the D7E3 and rabbit antiserum staining was controlled using mouse monoclonal antibody of the same subclass (IgG_1, MOPC 21 from Letton Bionetics Inc., Kensington, USA) and a non-specific rabbit antiserum (Sigma). In each experiment, MCF7 cells were used as positive stained control. They were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 80% confluence. They were cytospun and stored at −20°C in 250 mmol/l sucrose glycerol, PBS (vol/vol) after fixation in 3.7% formaldehyde.

Quantification by image analysis

A computerised image analyser (SAMBA 2005 TITN Alcatel Grenoble, France) was used to quantify staining. This system is a recent version of the SAMBA 200 TITN [14] and includes a JVC, KY-15, 3-CCD video camera mounted on a BH-2 light microscope (Olympus) and connected to a microcomputer. Two frozen sections taken from different parts of the tumour were analysed to take tumour heterogeneity into account. Only staining in tumour cells was selectively quantified. The counterstained surfaces were measured to determine the percentage of stained tumoral cell surface. In order to correlate results of immunostaining and of cytosolic assay, we estimated the averaged staining intensity rather than the degree of staining heterogeneity in one section. Staining intensity was averaged from integrated optical densities of three different fields corresponding with an average of 450 to 600 tumoral cells per slide at ×40 final magnification. This technique of quantification was previously validated [15]. In each experiment, cytospun MCF7

cells were used as positive stained control, their staining being constant (SD 10%): no corrections were applied.

We averaged only three stained fields in each frozen section, since there was no significant change when more fields were analysed. Staining intensity of control slide was subtracted from the intensity obtained with the specific antibody. Results were expressed by a quantitative immunochemical (QIC) score [6] [=(percentage of surface stained in epithelial cells) × (mean staining intensity) × 10].

RESULTS

Histological characterisation

34 specimens (out of 42 analysed) contained invasive breast carcinoma tissue (31 were ductal and one was lobular). Tissue cath-D was quantified in 16 benign juxta tumoral tissue samples. Eight specimens contained only peritumoral tissue and eight sections contained both tumoural and non-malignant breast tissues. Non-tumoral tissue comprised four normal mammary glands, and 12 cases of fibrocystic disease. In the last group, seven had ductal hyperplasia and two had lobular hyperplasia. Cath-D staining was quantified in the whole population using D7E3. In 25 breast cancers, cath-D was immunostained in serial frozen sections using both D7E3 mouse monoclonal and rabbit polyclonal antibodies.

Immunohistochemical staining of cathepsin D using D7E3 antibody

Cath-D immunostaining in frozen sections was cytoplasmic, not punctuated and mostly observed in cancerous cells (Fig. 1a). Some stromal and normal mammary glandular cells were faintly stained in some sections [Fig. 1(c)]. Macrophages and histiocytes were stained but not monocytes; however, their proportion was less than 15% of the total counted cells.

Results on quantification of staining intensity using a computerised image analyser were reproducible. Two independent observers obtained similar results, as determined using a t-test for paired samples ($P = 0.55$). Using the D7E3 antibody, QIC score values were highly dispersed in the 34 cancer samples (range 0.5–421 AU) (Fig. 2). We obtained an abnormal distribution and the mean value was significantly higher than in non-malignant breast tissue [mean (SD) 90.5 (94.5) AU vs. 220.2 (54.6) AU]. High cath-D values (222 AU) were obtained in only one juxta-tumoural tissue, a fibrocystic disease without other hyperplasia or cellular atypia, while adjacent ductal carcinoma showed weak cath-D staining (6 AU). Macrophages were generally highly stained but their numbers (evaluated by specific antibodies) were generally low relative to the whole tumour cell population. In one tumour, characterised by an absence of immunostaining in the tumour cells but with a high percentage (>13%) of cath-D containing macrophage-like cells, the cath-D level was found to be low (21 pmol/mg protein) in the cytosol.

Correlation between cath-D levels using mouse D7E3 monoclonal and rabbit polyclonal antibodies

In cancer cells, there was no difference between mean levels of cath-D staining using rabbit polyclonal antibodies or D7E3 antibody (Figs 2, 3). In the 25 cancer specimens analysed with the two antibodies, the average QIC scores were 92.4 and 83.5 AU for D7E3 and polyclonal antibody, respectively (Fig. 3) ($r = 0.53$; $P < 0.0001$). The major difference between the two antibodies was a higher staining of non-tumoral mammary glands by the polyclonal antibodies than by the D7E3 antibody (Figs 1, 2). Equivalent QIC scores of cath-D staining were observed in malignant and non-malignant cells when using polyclonal antibodies (Fig. 2).

Correlation between cath-D levels using tissue immunostaining and cytosolic assay

In the same population of 25 invasive ductal breast carcinomas, the quantitative immunohistochemical cath-D score was found to be correlated with its cytosol concentration whether D7E3 antibody ($r = 0.80$) or polyclonal antibodies ($r = 0.66$) were used (Fig. 4). The better correlation observed with D7E3 was mostly due to the continuous increase of staining at high cath-D concentrations.

Using polyclonal antibodies, the QIC score more rapidly reached a maximum, but did not increase at concentrations higher than 100 pmol/mg protein. In these patients, we used a cut-off level of 50 pmoles/mg cytosol protein (close to the median value) to separate 50% of the breast cancer cases according to low or high cytosolic cath-D status (Table 1). The immunohistochemical 48% cut-off levels were 60 AU for D7E3 antibody and 43 AU for the polyclonal antibodies. However, a better overall correspondence between the two techniques was obtained with a cut-off level of 50 AU for both antibodies. In these conditions, sensitivity was 84% and specificity was 77% using D7E3 antibody and 75 and 85%, respectively, using rabbit polyclonal antibodies. In the same conditions, with the D7E3 antibody, there were only 2 patients out of 25 with higher cytosol values and 3 patients out of 25 with higher immunohistochemical values. Using the same cut-off level, the discrepancy was similar with the polyclonal antibody (3 and 2 out of 25 for both cases).

DISCUSSION

The correlation between high cath-D level in breast cancer cytosol and higher frequency of relapse and metastasis within 5–6 years after surgery has been demonstrated in several independent retrospective studies (for review, see [4]). By contrast, the only immunohistochemical study available indicated a good prognostic value of cath-D staining in cancer cells [8]. However, in this study different antibodies were used on paraffin embedded sections and there was no quantification of staining intensity. In this first quantitative study of cath-D level by immunohistochemical analysis of frozen sections, we show that a large range of cath-D concentrations can be quantified in breast cancer cells using a computerised image analyser. When taking a cut-off level of 50 AU to discriminate high and low cath-D staining, similar correlations with the cytosol assay were obtained whether the D7E3 mouse monoclonal antibody or the rabbit polyclonal antibodies were used. However, by immunohistochemistry, there were approximately 10% of patients with lower values and approximately 20% of patients with higher values than with the cytosol assay. This variation might be due to tumour heterogeneity.

In this study, only staining in tumour cells was quantified. D7E3 staining in fibroblasts and endothelial cells surrounding cancer tissue was negligible. However, macrophages and histocytes were also stained for cath-D but accounted for less than 15% of cells, as estimated using anti CD68 antibodies (Dako). Accurate quantification of macrophages might be difficult, however, since most of the available antibodies also recognized antigens in mammary tumour cells [16, 17]. In one case where the estimated proportion of cath-D positive macrophages was relatively high (13% of total cells) and cath-D staining was low in cancer cells, cytosolic cath-D level was also low (22 pmoles/mg protein). Using the D7E3 antibody and quantitative image analysis, we also confirmed that cath-D levels were generally higher in malignant cells than in normal glands or benign mastopathies adjacent to the tumour. This confirmed previous

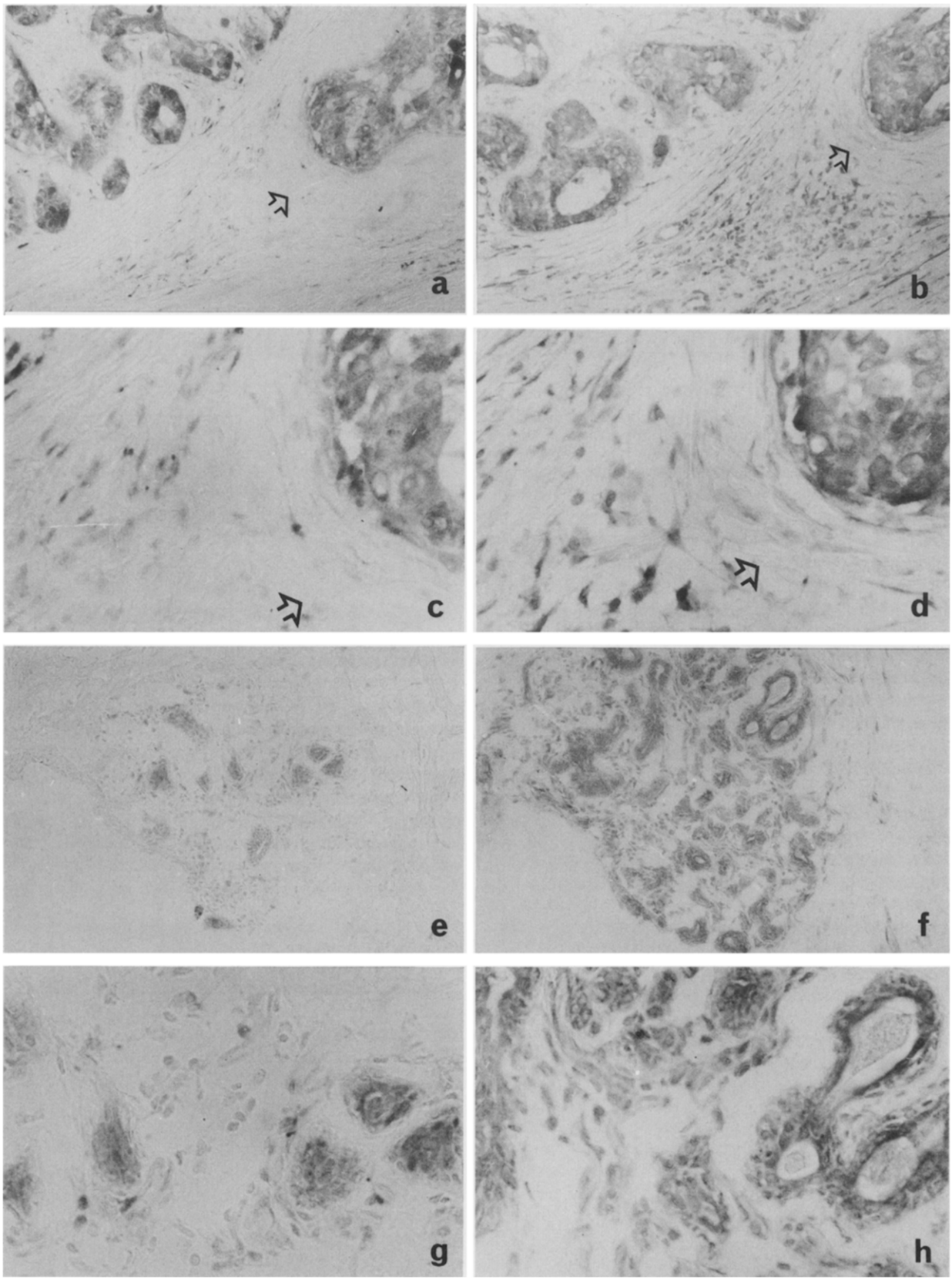

Fig. 1. Cath-D immunostaining of breast cancer [(a)–(d)] and normal mammary lobules [(e)–(h)] without counterstaining. (a) and (b) are serial frozen sections of an invasive ductal carcinoma (×20). (c) and (d) are the same sections at higher magnification (×60). (e) and (f) are serial frozen sections of peritumoral tissue of the same breast cancer (×20). (g) and (h) are the same section at higher magnification (×60). Mouse monoclonal antibody D7E3 [0.1 μg; left column (a), (c), (e) and (g)] and rabbit polyclonal antibodies [1/300; right column (b), (d), (f) and (h)] to human cath-D were used as described in Materials and Methods and show similar staining for breast cancer but a much higher staining of non-tumoral glands with the polyclonal antibodies. Arrows indicate area of stromal cells.

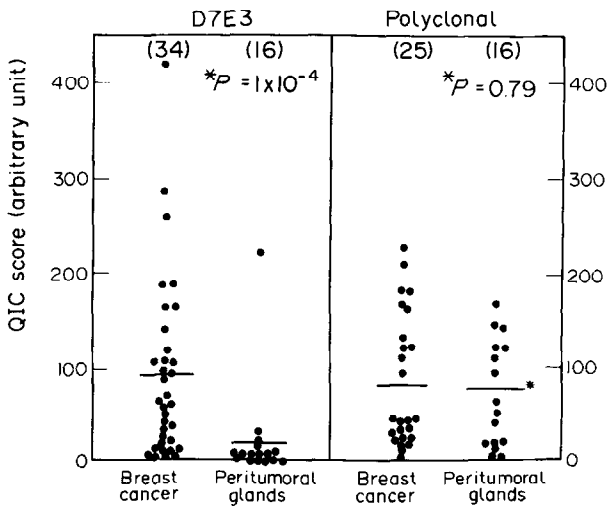

Fig. 2. Immunohistochemical quantification of cath-D staining (QIC score) in breast cancer and peritumoural glands using D7E3 monoclonal antibody and polyclonal rabbit antibody. Horizontal bar represents the average of each group. Statistical significance was determined using non-parametric Wilcoxon and Mann–Whitney tests (*P*). Number of cases in each group is shown in parentheses.

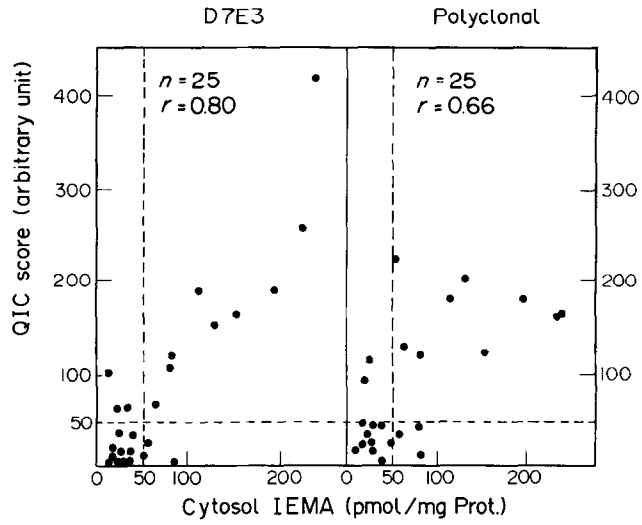

Fig. 4. Correlation between cytosolic cath-D by IEMA and QIC score of cath-D staining using D7E3 or polyclonal rabbit antibodies in the same 25 breast cancer tumours. The significance of the coefficient of correlation (*r*) was tested using a *t*-test. Dotted lines represent an arbitrary cut-off level of 50 pmol/mg protein and 50 AU which showed the best correlation between the biochemical and the two histological assays (see Table 1).

clinical and biological [4, 5] results showing increased gene expression of cath-D in breast cancer cells compared with normal cells.

In the first immunohistochemical study from our laboratory, in which the D7E3 antibody was used at a higher concentration (10 μg/ml), there was no staining in normal mammary glands but homogeneous staining was observed in some benign mastopathies associated with ductal hyperplasia or large cysts [18]. In this study, only the percentage of stained cells was estimated, not the concentration. The advantage of computerised quantification compared to visual analysis is that objective and complete quantification of the staining is obtained, thus the sensitivity and accuracy of the assay is increased and a prognostic cut-off level can be determined.

There are several possibilities to explain the different results obtained by Henry *et al.* concerning the prognostic value of cath-D. Firstly, the antibodies are different and their affinity for the antigen may also be different since they were raised against different antigens. For instance, the staining in non-tumoral mammary glands appears to be higher with the polyclonal antibodies. Secondly, the technique of Henry *et al.* is only semi-quantitative and in our conditions, the polyclonal antibodies gave a staining saturation for cath-D concentrations

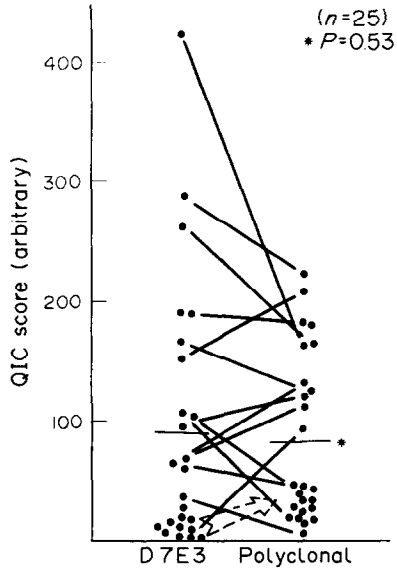

Fig. 3. Immunohistochemical quantification (QIC score) of cath-D staining in 25 breast cancers using D7E3 monoclonal antibody and rabbit polyclonal antibodies. Serial sections of the same specimen stained with D7E3 and polyclonal antibodies are connected. The arrow represents the connections between the mean of low QIC score values. Statistical significance was determined using non-parametric Wilcoxon and Mann–Whitney tests. It is not different for the mean but the range is higher for D7E3.

Table 1. Comparison of cut-off levels to stratify breast cancer population according to cath-D concentration estimated by three different techniques

Antibody	QIC score (AU)	Repartition of patients according to cytosol Cath-D status +	−	% of cath-D negative
D7E3	≥60	10	3	
	<60	2	10	48
Polyclonal	≥43	9	4	
	<43	3	9	48
D7E3	≥50	10	3	
	<50	2	10	48
Polyclonal	≥50	9	2	
	<50	3	11	56

Classification of breast cancer according to cath-D level determined in cytosol and by two quantitative immunohistochemistry analyses using a threshold value of 50 pmoles/mg of cytosol protein (IEMA) and the indicated immunohistochemical QIC score values expressed in arbitrary unit (AU).

higher than 100 pmol/mg protein (Fig. 4). Since the prognostic value of the cytosolic cath-D concentration depends upon the cut-off level chosen, the polyclonal antibodies might not be able to discriminate between moderate and high levels of cath-D.

Thirdly, a likely and biologically interesting explanation is that we do not measure the same antigens in the cytosol and in tissue sections and the source of high cath-D cytosolic concentrations might not be only breast cancer cells. In fact, immunohistochemistry mostly takes into account the antigen concentrated in intra-cellular compartments (lysosomes etc.), and may not detect the secreted pro-cathepsin D because of its larger diffusion or because it is washed out during the fixation procedure.

The major interest of immunohistochemistry as compared to the cytosolic assay is to define the cells which produce, or accumulate the antigens. Clearly, the major cath-D producing cells appear to be the cancer cells. Cath-D production by fibroblasts is very low compared to cancer cells as shown by immunohistochemistry and *in situ* hybridisation with antisense RNA (C. Escot *et al.*INSERM, Montpellier). Histiocytes and macrophages also produced high levels of cath-D. However, in non-inflammatory breast cancers corresponding to the tumour population analysed here, the percentage of macrophages was low compared to the amount of cancer cells extracted in a cytosol, and it is unlikely that they contribute much of the cath-D measured in the total cytosolic assay.

To conclude, cath-D distribution and concentration can be quantified in breast cancer tissue by computer assisted image analysis of frozen sections immunostained with D7E3 monoclonal antibody. There is a good correlation between this technique and cytosol IEMA, thus suggesting that the prognostic value of cath-D can also be determined by immunohistochemistry in small tissue samples, as previously verified for immunohistochemical ER analysis [19]. However, the correlation between the two techniques is not perfect, and a larger number of tumours will have to be analysed and clinical follow-up of patients will be required to determine the actual clinical significance of cath-D concentration in breast cancer tissue as determined by immunohistochemistry.

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