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Cathepsin D Assay in Primary Breast Cancer and Lymph Nodes: Relationship with c-*myc*, c-*erb*-B-2 and *int*-2 Oncogene Amplification and Node Invasiveness

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In breast cancer, axillary lymph node invasiveness is the major prognostic factor in predicting relapse and metastasis. Nevertheless, since 30% of node-negative tumors also relapse, it is necessary to develop other independent prognostic factors. Oncogene amplification and the level of cathepsin D (cath-D), an acidic lysosomal protease produced and secreted in excess by breast cancer cells, have been proposed as additional prognostic factors. We have compared the cytosolic cath-D level and the amplification of three oncogenes: c-*myc*, neu-*erb*-B-2 and *int*-2 in 140 primary breast carcinomas and 64 axillary lymph nodes collected in 1987 and 1988 at the Cancer Center of Montpellier (Centre Paul Lamarque). None of the patients had previously received hormonal or chemotherapy. The cath-D concentration was measured with an immunoradiometric assay using monoclonal antibodies. DNA purified from the same samples was analyzed by a standard Southern blotting technique to estimate oncogene amplification. No correlation was found between the level of cath-D in the tumor and node invasiveness. Using a cut-off level of 60 pmol/mg protein, the status of cath-D was not correlated with neu-*erb*-B-2 and *int*-2 amplification and only correlated with c-*myc* amplification ($P = 0.011$). Both c-*myc* and cath-D are associated with cell proliferation, induced by estrogens in ER+ breast cancer, and constitutively produced in ER− breast cancer. The level of cath-D was significantly higher in the invaded lymph nodes ($P = 0.04$) than in the histologically non-invaded ones. Nevertheless, some non-invaded lymph nodes contained a high level of cath-D, as confirmed by immunoperoxidase staining. In conclusion, in breast cancer, a high cytosolic cath-D concentration is more frequent in tumors with c-*myc* amplification but is dissociated from neu-*erb*-B-2 or *int*-2 amplification, suggesting that the determination of these three markers will have an additional prognostic value.
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INTRODUCTION

ONE of the major problems in the treatment of breast cancer is determining whether the disease is localized and cured at surgery by the removal of the primary tumor and lymph nodes or whether it is already generalized and therefore needs additional systemic hormonal or chemotherapy [1]. When patients are staged after primary surgery, the degree of lymph node invasion is currently the major prognostic factor, node-positive tumors generally implying high risk. On the other hand, about 30% of node-negative tumors also relapse and it has been proposed to apply systematic adjuvant therapy [2] to all patients, including those who are node-negative. This adjuvant therapy may, however, be harmful to patients who do not require it [3]. It is therefore crucial to use other prognostic factors that are independent of node invasiveness and can discriminate between high-risk and low-risk node-negative patients.

Several new prognostic factors have recently been proposed such as amplification and/or overexpression of oncogenes [4–6] as well as EGF receptor overexpression [7] and proteases such as cath-D [8] or plasminogen activator [9], in addition to the assay of estrogen and progesterone receptors [10]. Our laboratory has been engaged in studying the structure, function and regulation of a M_r 52,000 (52k) secreted protein induced by estrogen in ER-positive breast cancer cell lines [11]. This protein has been identified as pro-cath-D that is produced and secreted more abundantly by breast cancer cells than by normal mammary epithelial cells [12].

The first clinical studies using specific monoclonal antibodies to cath-D have shown that this marker, when assayed in breast cancer cytosol, is not correlated to Scarff and Bloom grading, tumor size, lymph node status, or estrogen or progesterone receptor content [13], since it is also produced constitutively by ER- and PR-negative cells. A 6–7-year retrospective study performed with the Danish Breast Cancer group and the Finsen

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Institute (both in Copenhagen) on 396 patients has shown that a high cath-D concentration in the cytosol of primary breast cancer indicates a poor prognosis independent of other prognostic factors [12, 14, 15]. The evaluation of independent prognostic factors is of prime interest since their predictive value is additive.

In the present study, we have measured by immunoradiometric assay total cath-D concentration in cytosol of 140 primary breast cancers, and compared it with the presence of c-*myc*, neu-*erb*-B-2 and *int*-2 oncogene amplification measured in the DNA of the same tumor. We also assayed cath-D in lymph node cytosol and correlated it with invasiveness as determined histologically by a pathologist, in order to determine whether node invasiveness not detected in tissue sections could be detected by cath-D assay in the corresponding cytosol.

MATERIALS AND METHODS

Tumor material

The study included 140 primary breast invasive ductal carcinomas without clinical metastasis and 64 axillary lymph nodes. All samples were collected within the last 2 years in the Pathology Department of the Cancer Center of Montpellier (Centre Paul Lamarque). None of the patients analyzed had received hormonal therapy prior to surgery. All surgical biopsies were snap-frozen within 2 h after surgery and stored at −80°C until processing.

Tumor sample processing

Frozen biopsy samples were ground to a fine powder in liquid nitrogen and subsequently homogenized in 20 mM Tris, 10 mM EDTA, 10 mM NaCl using a dounce homogenizer. The homogenates were spun at 800 ***g*** for 15 min at 4°C, yielding 800 ***g*** supernatants, which were quickly frozen and stored at −80°C. The pellets, containing cellular debris and nuclei, were processed for DNA extraction as previously described [16]. For cath-D measurements, the 800 ***g*** supernatants were thawed and ultracentrifuged at 100,000 ***g*** for 1 h at 4°C to obtain high-speed supernatants (cytosol) which were frozen at −80°C. Estrogen and progesterone receptors were assayed by the charcoal method as described [10].

DNA analysis

Tumor and normal control DNAs were analyzed by standard Southern blotting techniques. Seven μg was digested with either Eco RI or Hind III restriction endonucleases, fractionated on a 0.8% agarose gel and transferred onto Compas (Genofit) nylon membrane. Purified DNA probes were radioactively labeled (specific activity 1–3 × 10^9 cpm/μg of DNA) using the Amersham multiprime labeling kit. Hybridization conditions were those recommended by the nylon membrane manufacturer. After being washed under high stringency conditions, blots were exposed to Kodak XAR-5 or Amersham MP films with intensifying screens for 24–48 h at −80°C. The signal intensity of the bands was estimated by scanning the autoradiograms with a densitometer. The peak values of the hybridized probes were compared to the signals of control probes (c-*mos* and beta-globin as control for neu-*erb*-B-2 and c-*myc*, ETS1 and c-*mos* as control for *int*-2 and *erb*-A-2 as control for neu-*erb*-B-2) present on the same blot. Oncogenes were considered to be amplified when the ratio of the oncogene signal to the control signal was ≧2 as described in [A].

DNA probes

c-*myc*: 0.9 kb Pst I fragment from p-*ryc* 7.4 clone [18]; neu-*erb*-B-2: 1.6 kb Eco-RI fragment from p1622 clone (kindly provided by Dr M.P. Simon); *int*-2: 0.9 kb Sac I fragment of

Table 1. *Distribution of patients according to cathepsin D status (low or high) and other prognostic parameters*

			Patients with low cath-D (<60 pmol/mg)		Patients with high cath-D (≧60 pmol/mg)	
		P value	*n*	Percentage positive	*n*	Percentage positive
neu-*erb*-B-2 amplification						
(22.9%)	+	0.55	17	22.7	15	23.1
	−		58		50	
c-*myc* amplification						
(22.9%)	+	0.011	11	14.7	21	32.3
	−		64		44	
int-2 amplification						
(13.6%)	+	0.20	8	10.7	11	16.9
	−		67		54	
Node invasiveness						
(55%)	+	0.34	32	45.7	31	50.8
	−		38		30	

Oncogene status, defined as non-amplified (−) or amplified (+), was determined in 140 patients.

The percentage of oncogene amplification and node invasion in this study is shown in brackets. Node status, invaded (+) or not (−), was known in only 131 patients. The cut-off level for cath-D was taken as 60 pmol/mg cytosol protein which corresponds to the upper limit of the median class of concentrations. Probabilities were determined using Fisher's exact test.

n = number of patients.

the pSS6 clone [19]; c-*mos*: 2.5 kb Eco-RI fragment (ATCC No. 41004); beta-globin: 1.4 kb Eco-RI to Hind III fragment of the HB 6 clone [20]; *erb*-A-2: 1.9 kb Eco-RI fragment of pH-c-*erb*-A-2 clone [21]; ETS1: 0.75 kb Hind III fragment from clone pHE 5.4 [22].

Cathepsin D immunoassay

A one-step double determinant solid-phase immunometric assay previously described [13, 23] 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) radiolabeled with ^{125}I. The assay was performed on diluted cytosols of breast cancer or lymph nodes (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 stiring up. The tubes were then washed and counted in a Packard gamma scintillator, adjusted for ^{125}I measurements. Cathepsin D concentration is expressed in pmol/mg cytosol proteins assayed by the Bradford technique (Bio-Rad Laboratories Gmb, Munchen, F.R.G.) using bovine serum albumin as the standard.

Immunohistochemical staining of cathepsin D

Frozen sections 6 µm thick, fixed in colored acetone, were stained by the Steinberger peroxidase–antiperoxidase method, as previously described [24] using 100 µl/slide of a 10 µg/ml M1G8 antibody solution. A negative control was performed with a nonrelevant monoclonal antibody (IgG1: MOPC 21, Bionetics) of the same subclass.

Statistical methods

Statistical differences within the population were determined by the Kruskall–Wallis or Mann–Whitney non-parametric tests for quantitative parameters. The χ^2 test and Fisher's exact test were used for qualitative parameters. Linear regression was calculated by Pearson's least squares method. Pearson's correlation coefficients were analyzed by Student's *t* test.

RESULTS

Validity of cathepsin D radioimmunoassay RIA

Cytosols from 119 breast cancers or nodes were assayed both by RIA with the ELSA-cath-D kit and by the ELISA method previously described [12, 23] (Fig. 1). The correlation coefficient calculated by linear regression was 0.985. No difference was found between the mean values of the two assays (29.08 pmol/mg protein for RIA and 29.10 pmol/mg protein for ELISA). However, the ELSA-cath-D assay showed a better range of between-assay coefficients of variation (from 2.5 to 5.3%) than the ELISA method.

Characteristics of the population

In 140 primary breast cancers, the cytosolic cath-D concentration on the one hand and c-*myc*, *int*-2 and neu-*erb*-B-2 oncogene amplifications on the other hand were measured blindly in two different laboratories without knowledge of the other parameter. Axillary lymph nodes were histologically invaded (N+) in 77 patients (58.7%). Node status was unknown in nine patients. The mean concentration of cath-D in the cytosol was 82.4 ± 22.8 pmol/mg protein (mean ± S.D.). The

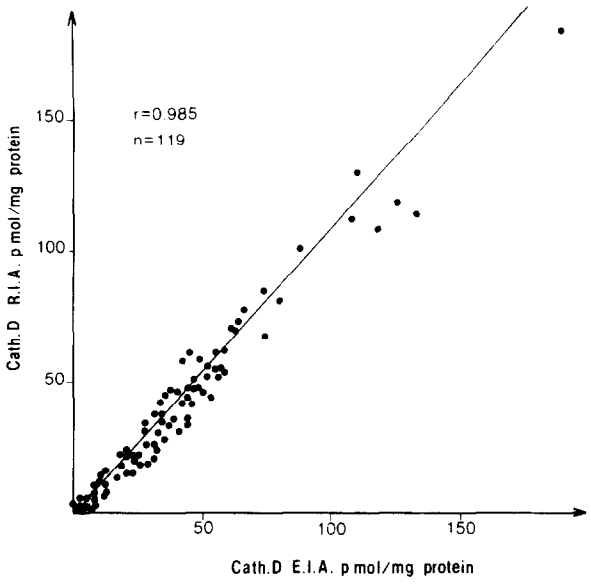

Fig. 1. Correlation between ELSA-cath-D RIA and cath-D EIA. Cytosols (119) were assayed for cath-D using the previously described EIA [13, 23] and the new ELSA-cath-D RIA described in Materials and Methods. The correlation rate (*r*) was highly significant (*P* = 0.001) according to Student's *t* test. The mean concentration was nearly identical in the two assays (29.08 pmol/mg P in RIA and 29.10 pmol/mg P in ELISA).

distribution favored tumors with low cath-D concentrations (Fig. 2), the median class value was 50–60 pmol/mg protein. The incidence of amplification in this breast tumor population was of 22.9%, 22.9%, 13.6% for c-*myc*, neu *erb*-B-2 and *int*-2 respectively.

Cathepsin D and oncogene amplification

The cath-D concentration in breast cancer cytosol was compared to the presence or absence of oncogene amplification. No significant correlation was found between neu *erb*-B-2 or *int*-2 oncogenes versus cath-o status (Table 1, Fig. 3). The only correlation was between c-*myc* oncogene amplification and high cath-D status (*P* = 0.011). In tumors with low cath-D status (concentrations <60 pmol/mg cytosol protein), the proportion of c-*myc* amplification was lower (14.7%) than in tumors with high (>60 pmol/mg protein) cath-D status (32.3%) (Table 1). Whereas, tumors with or without c-*myc* amplification contained

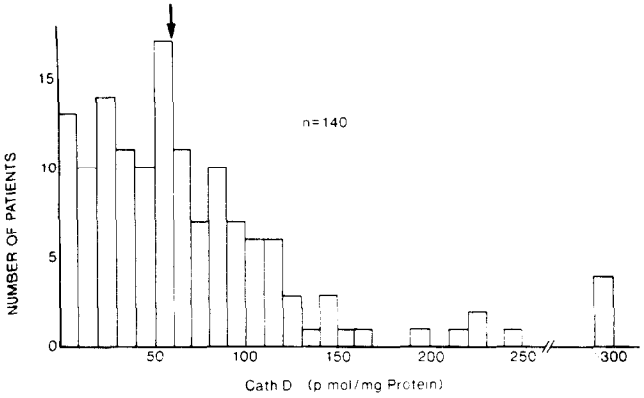

Fig. 2. Distribution of primary breast cancers according to the cathepsin D concentration in cytosols measured by immunoradiometric assay. The class interval is 10 pmol/mg protein. The median class is between 50 and 60 pmol/mg protein. The arrow indicates the cut-off level between high and low cath-D concentrations.

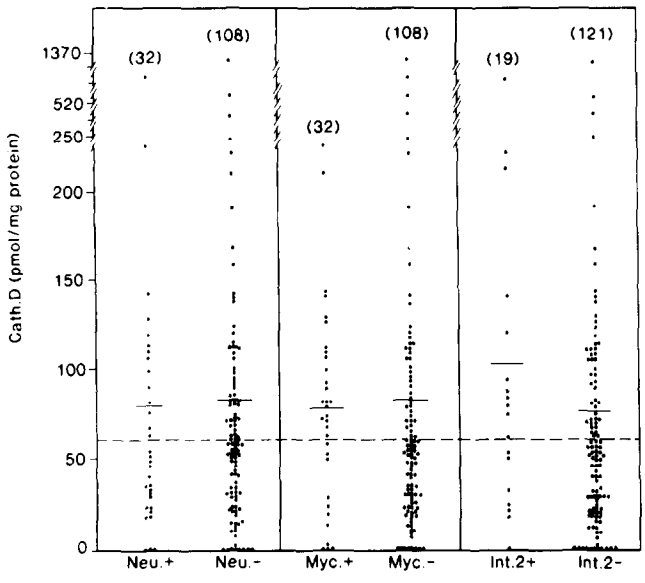

Fig. 3. Relationship between cathepsin D concentration and oncogene amplification. The cath-D concentration in 140 primary breast cancer cytosols are plotted according to the presence (+) or absence (−) of neu-*erb*-B-2, c-*myc* or *int*-2 gene amplification. There is no statistically significant difference between the six groups in the mean cathepsin D concentration and the presence or absence of oncogene amplification, according to the Kruskall–Wallis non-parametric test. The only significant correlation is between c-*myc* and cath-D status, taking a cut-off level of 60 pmol/mg protein (indicated as a broken line) (see Table 1).

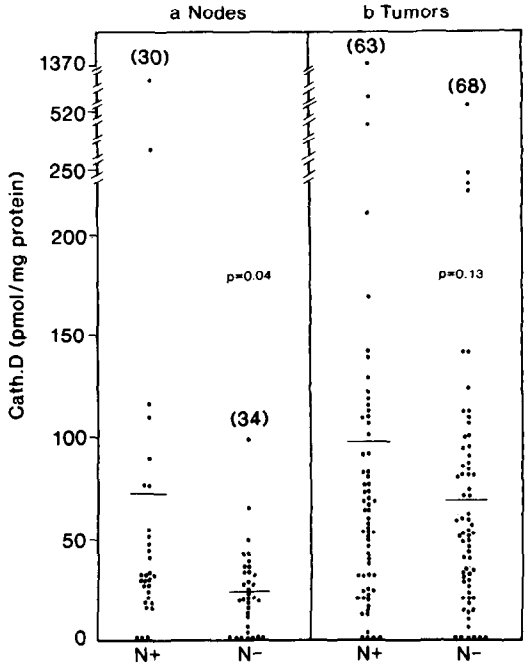

Fig. 4. Relationship between cathepsin D concentration and node invasiveness. The cath-D concentration in 64 cytosols of axillary lymph nodes (a) and 131 primary tumors (b) is plotted according to the axillary lymph nodes status as staged in parallel by a pathologist. (N+) invaded, (N−) non-invaded. The difference is only significant for cath-D level in lymph nodes (Mann–Whitney non-parametric test).

similar mean levels of cath-D (Fig. 3). We confirmed previous reports [8, 13–15] on the absence of correlation between the cath-D level and estrogen receptor or progesterone receptor status (not shown). Some patients had two simultaneously amplified oncogenes, nine with both c-*myc* and neu-*erb*-B-2, four with both neu-*erb*-B-2 and *int*-2 and four with both c-*myc* and *int*-2.

Cathepsin D and lymph node invasiveness

The absence of correlation between the cytosolic cath-D concentration in primary tumors and lymph node status [13–15] was also confirmed (Table 1, Fig. 4b). However, the cath-D concentrations differed significantly ($P = 0.04$) in the cytosols of invaded lymph nodes and non-invaded lymph nodes, as defined histologically (Fig. 4a), in agreement with a higher cath-D concentration in breast cancer cells than in lymph node cells [24]. Using immunoperoxidase staining with the monoclonal antibody M1G8 [25], we generally detected positive staining in invaded nodes, and no staining in negative nodes. In two cases, we also found positive cath-D staining in the lymph nodes with high cytosolic cath-D level which was however found to be negative histologically (not shown). However, different parts of the nodes may have been analyzed and further studies are needed before to conclude that cath-D staining is a more sensitive technique than the current histopathological examination to detect lymph node invasion.

DISCUSSION

Among the prognostic markers assayed in the primary breast cancer following surgery, those providing independent information are of particular interest. The present study confirms and complements previous studies assessing the clinical value of cath-D in breast cancer cytosol. From retrospective studies [12, 14, 15], this marker appears potent in predicting relapse-free survival. It is independent of classical prognostic markers such as lymph node invasiveness, tumor size, Scarff and Bloom stage, estrogen receptors, progesterone receptors, age of patients.

In the present study, we show that cath-D level is also independent of neu-*erb*-B-2 and *int*-2 oncogene amplification, but is correlated with c-*myc* amplification. c-*myc* expression is generally related to cell proliferation [25, 26], and gene amplification in breast cancer has been shown to be correlated to RNA overexpression [27]. Moreover, it has been reported that breast cancer patients whose tumor DNA shows an amplified c-*myc* gene have a decreased short-term survival [28]. We have currently no explanation for the correlation between c-*myc* alteration and cath-D levels. It might be, however, related to the fact that both markers are induced by estrogens in estrogen-receptor-positive (ER) breast cancer cell lines [11, 29] and expressed constitutively in ER-negative breast cancers.

Since cath-D concentration is not correlated with neu-*erb*-B-2 amplification, it may be of interest to assay these two markers in the same tumor, since their prognostic value would be additive. Neu-*erb*-B-2 gene amplification is correlated with the absence of estrogen and progesterone receptors [30, 31] and with poor short-term prognosis mostly in node positive patients [4, 28]. By contrast, cath-D appears to be an independent marker that is mostly useful in node-negative patients indicating that the information given by these two markers is complementary. *Int*-2 amplification appears to be independent of other clinico-pathological parameters in breast cancer [5, 32, 33] and to be correlated with the probability of distal metastasis [34]. Therefore, cath-D and *int*-2 amplification might also constitute two independent parameters with additional prognostic values.

In practice, we show here that a convenient homogenization procedure can be used to assay estrogen receptors, progesterone

receptors and cath-D in the cytosol of the same tumor sample, and to determine oncogene amplification for at least three different oncogenes in the remaining pellet.

The information concerning the presence and level of cath-D in axillary lymph nodes would require additional studies. Since, in multiparametric Cox analysis, lymph node status is one major prognostic marker, it is important to improve its reliability. The correlation between histological lymph node invasiveness and high cath-D concentration is consistent with the higher cath-D concentration in breast cancer cells than in non-invaded lymph nodes or fibroblasts. A high cathepsin D level in lymph node cytosol may therefore contribute to detecting false node-negative breast cancers. However, further studies are required before one can propose that the classical histopathological examination of lymph nodes to detect invasive breast cancer cells should be completed by immunohistochemical staining of cath-D [24] or other specific antigens of epithelial cells (keratin) [35] or mammary cells (milk fat globule antigens) [36].

It is likely that, within the next decade, an increasing number of prognostic markers including oncogenes and proteases will have to be classified into categories and ranked by multiparametric studies to determine those that have the highest predictive value.

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