

Prognostic value of cathepsin D in breast cancer: comparison of immunohistochemical and immunoradiometric detection methods

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Abstract

Aim—To test whether immunoradiometric or immunohistochemical detection of lysosomal protease cathepsin D in breast cancer is more predictive of outcome.

Methods—Tumour tissues from 270 primary breast cancer patients were evaluated for the expression of cathepsin D using immunohistochemistry (IH; paraffin embedded tissues) and an immunoradiometric assay (IRMA; cytosol from frozen tissues). Immunohistochemical scores were based on immunoreaction in tumour cells and tumour associated macrophages.

Results—IRMA values (cut off 40 fmol/mg cell protein) correlated significantly with IH values. Recorded incidences of positive immunoreaction in tumour cells using two different cut off values were 52% and 35%, respectively. Macrophages stained positive in 31% of tissues. Combined evaluation of tumour cells and macrophages resulted in positivity rates of 59% and 48%, respectively. Node status was the only variable found to correlate with cathepsin D expression. IH results correlated significantly with clinical outcome (median observation time 68 months) in node negative patients (n=120) but not in node positive patients (n=145). Cathepsin D positivity as measured by IRMA was not related to clinical outcome in either group. On multivariate analysis in the node negative group, IH detection of cathepsin D appeared to be the only independent factor indicating prognosis. For node positive patients, tumour grade, size, and receptor status were of prognostic relevance.

Conclusions—Because of the simple methodology and the minimal amount of tissue used for analysis, immunohistochemistry was preferred to immunoradiometry for cathepsin D measurement; it also provided more predictive data with respect to prognosis.

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Keywords: cathepsin D, breast cancer, immunohistochemistry, immunoradiometric assay, prognosis.

In 1979 Westley and Rochefort¹⁵ described the purification of a glycoprotein fraction (cathepsin D) from oestrogen stimulated, oestrogen receptor (ER) positive MCF-7 breast cancer cells. In cells of the mammary gland the enzymatically inactive procathepsin D (M_r 52 kDa) is completely transformed into an intermediate product (M_r 48 kDa) consisting of the mature forms (M_r 34 kDa and M_r 14 kDa).^{16,17} Only minimal amounts of procathepsin D are accumulated in benign epithelial cells,^{5,17} whereas carcinomatous tissues contain much higher levels.^{5,18-20} The overexpression of cathepsin D precursors causes an overloading of the lysosomal transport system. More procathepsin D is thereby secreted into the cytoplasm and out of the tumour cell.^{1,17,18} The human cathepsin D gene is located at the outer end of chromosome 11p close to the *harras* proto-oncogene.²¹

At present cathepsin D is measured mainly by immunoradiometric assays (IRMA) in tumour cytosols^{5-7,9,10,12,13,22-25} requiring fresh or deep frozen (-70°C) tissue. Routine monitoring requires a method by which cathepsin D can be measured reliably and reproducibly in minute amounts of routinely processed tissues, as is the case in immunohistochemistry (IH). Recently some investigators,^{11,22,25-28} including ourselves,²⁹ compared the results of cathepsin D detection using both these methods and reported concordance rates of approximately 70%. However, a 30% discordance of results may make a difference when prognostic information is required.

The retrospective study reported here compares immunoradiometric measurement of cathepsin D in deep frozen tissue cytosols with immunohistochemical detection in formalin fixed and paraffin embedded surgical specimens of 270 primary breast carcinomas for their predictive value with regard to the clinical course of the disease. The aim was not to evaluate concordance rates but to assess the predictive potential of either method. On the basis of a subtle scoring system we provide evidence that the predictive value of immunohistochemistry may be superior to that of immunoradiometry.

Methods

PATIENT SPECIMENS

We tested tissue specimens of primary, invasive, ductal breast carcinomas from 270 patients who had been treated surgically between 1983 and 1988 at the Department of Obstetrics and Gynaecology of the University of Cologne.

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Proteases are involved in neoangiogenesis, invasive growth, and metastasis of carcinomas.¹⁻³ The overexpression of the lysosomal protease cathepsin D is currently being investigated as a prognostic marker in node negative breast cancer patients.⁴⁻¹⁴

Local treatment consisted of simple mastectomy (n=171) or lumpectomy (n=99) plus postoperative irradiation of the breast (linear accelerator: 50 Gy to the whole breast and a boost of 10 Gy to the tumour bed). In 265 patients, axillary lymphadenectomy was performed up to level II, removing at least 10 nodes. No adjuvant treatment was given to node negative patients. Node positive, steroid receptor positive postmenopausal patients received adjuvant tamoxifen therapy (20–30 mg/day for two to three years). Node positive premenopausal women, and node positive, receptor negative postmenopausal women received six cycles of adjuvant chemotherapy (cyclophosphamide/methotrexate/fluorouracil or epidriamycin/cyclophosphamide). Follow up examinations were performed at regular intervals by our outpatient service or by co-operating gynaecologists in private practice. At present the median follow up period is 68 months (range 61–138 months).

Histological subtyping of tumours followed WHO guidelines (1981). Tumours were staged according to the TNM system of UICC. The histological grading was assessed according to the recommendations of Bloom and Richardson. Oestrogen receptors and progesterone receptors (PR) were detected and evaluated immunohistochemically as described previously³⁰ (ER: ERICA, Abbott; PR: mPR1, Dianova).

IMMUNOHISTOCHEMISTRY

Immunohistochemical detection of cathepsin D was based on the reactivity of monoclonal mouse antibody (His-Cath-Ab1: M1G8, Isotopen Diagnostik CIS) and a modified avidin-biotin complex method described by Hsu *et al.*³¹ Specificity of the antibody was checked by western blot analysis: immunoreactive bands were located at the 52 kDa, 48 kDa, and 34 kDa positions (procathepsin D, intermediate and mature form). Reproducibility of immunohistochemical detection was tested by repeated staining of serial sections from 35 tumour specimens which produced nearly identical results. Positive and negative controls were used throughout each staining procedure. Macrophages were identified by use of a specific antibody (CD68).

Sections of 3–4 µm thickness cut from routinely processed, formalin fixed (for up to 24 hours in neutral buffered formalin), paraffin wax embedded tumour tissues were mounted on glass slides, deparaffinised (30 minutes), and rehydrated in descending alcohol concentrations. After enzymatic digestion with trypsin (0.1%, 15 minutes) the slides were incubated with primary antibody (1:50), bridging antibody (antimouse IgG 1:100, Vectastain BA 2000), and avidin-biotin complex (1:100, Vectastain Elite ABC-Kit PK 6100) for 30 minutes each in a moist chamber at room temperature. Between incubations slides were rinsed with phosphate buffered saline (PBS). Antigen-antibody complexes were visualised using diaminobenzidine (12 minutes). Hemalaun was used for counterstaining (two minutes).

Immunoreaction was assessed independently by two of us (U-JG, AS). Where discrepancies occurred results were re-evaluated and discussed until a final agreement was reached. Immunoreactivity of tumour cells (immunoreactive score=IRS) was assessed by multiplying the percentage of positive cells (no positive cells=0, <10%=1, 10%–50%=2, >50%=3) by staining intensity (weak=1, moderate=2, strong=3), which produced a 10 point scale (IRS 0–9).

Immunoreactivity of tumour stroma (reaction=R) was evaluated on a four point scale ranging from 0 to 3 (0=no positive stromal cells; 1=single stromal cells stained; 2=combined staining of several stromal cells; 3=strong reaction). In order to find the most appropriate method of assessing immunohistochemical measurements of cathepsin D, tumours were qualitatively characterised as positive or negative using different cut off levels and varying combinations of immunoreactivity in tumour cells and stroma (table 1). These results were individually compared with the results of the immunoradiometric assay, related to clinical outcome (table 2), and then analysed statistically.

BIOCHEMISTRY

Cathepsin D concentrations were measured in 270 deep frozen tumour cytosols (–70°C) which had been prepared for steroid hormone receptor determination between 1983 and 1988. However, in one case the amount of cytosol remaining was insufficient for cathepsin D measurement. The tumour tissues used for cytosol extraction and for immunohistochemistry, respectively, were adjacent parts of the same tumour sample. Tissue processing and cytosol extraction were performed according to the EORTC guidelines.³²

Cathepsin D concentrations in cytosols were measured by an immunoradiometric assay (ELSA-CATH-D, Isotopen Diagnostik CIS) using a monoclonal antibody that recognises 52 kDa procathepsin D and its 48 kDa and 34 kDa secretion products. Cathepsin D concentrations (fmol/ml) were related to cytosol protein concentrations (0.9–10.4 mg/ml) measured according to Lowry *et al.*,³³ and recorded in pmol/mg. The minimum detection limit of the assay was 200–300 fmol/ml. The recovery rate ranged from 96% to 109% (540–2540 fmol/ml). The coefficients of the intra-assay (n=4 assays, 21–103 pmol/mg) and interassay variance (n=13 assays, 23–118 pmol/mg) were 3.8% and 10.1%, respectively. A cathepsin D cytosol concentration of 40 pmol/mg was taken as the cut off limit.⁵

STATISTICS

Statistical analyses were performed using the software SPSS 5.0.2 for windows (Statistical Package for the Social Sciences; Munich). The Spearman rank test was used for comparison of the immunohistochemical and immuno-

Table 1 Comparison of cathepsin D detection by immunoradiometric assay (IRMA) and by immunohistochemistry (IH)

IH	n	IRMA	IRMA	Concordance
		<40 pmol/mg n 116 (43%)	≥ 40 pmol/mg n 153 (57%)	
Evaluation 1 (E1)				
negative (tumour cells IRS=0)	130 (48%)	75	55	64% r=0.66 p<<0.001
positive (tumour cells IRS ≥ 1)	139 (52%)	41	98	
Evaluation 2 (E2)				
negative (tumour cells IRS ≤ 1)	174 (65%)	98	76	65% r=0.64 p<<0.001
positive (tumour cells IRS ≥ 2)	95 (35%)	18	77	
Evaluation 3 (E3)				
negative (stroma cells R ≤ 1)	185 (69%)	105	80	66% r=0.65 p<<0.001
positive (stroma cells R ≥ 2)	84 (31%)	11	73	
Evaluation 4 (E4)				
negative (IRS=0 and R ≤ 1)	109 (41%)	69	40	68% r=0.68 p<<0.001
positive (IRS ≥ 1 and/or R ≥ 2)	160 (59%)	47	113	
Evaluation 5 (E5)				
negative (IRS ≤ 1 and R ≤ 1)	141 (52%)	92	49	73% r=0.69 p<<0.001
positive (IRS ≥ 2 and/or R ≥ 2)	128 (48%)	24	104	

radiometric data. Univariate analyses utilised the χ^2 test. Follow up evaluations were based on log rank test³⁴ and graphically visualised by Kaplan-Meier curves. Multivariate analyses used the Cox proportional hazards model by calculating relative risks.³⁵

Results

Immunohistochemistry using a monoclonal mouse antibody (M1G8) on formalin fixed, paraffin embedded primary breast cancer tissues visualised cathepsin D as an intracytoplasmic granular staining in tumour cells and in “tumour infiltrating macrophages” (stromal cells) (for microphotographs see Göhring *et al*²⁹). Staining of tumour cells tended to be more intense towards the cell membranes. There was little intratumoral variation of staining intensity in positive tumour cells, whereas

stromal cells varied considerably in this respect. Non-neoplastic peritumoral lobular or ductal epithelia showed no specific staining. The co-existence of cathepsin D positive tumour and stromal cells within a tumour was statistically highly significant (p<<0.001). In only 7% of the examined tissues did we fail to observe any parallels between macrophage positivity and tumour cell immunoreactivity. The distribution of positive cells within tumour tissue was heterogeneous. By using evaluation formulae E1 (IRS 1) and E2 (IRS 2), 52% and 35% of tumour tissue specimens were denoted positive. Immunoreaction in stromal cells (E3: R 2) was seen in 31% of tumours, while evaluations E4 (IRS 1 and/or R 2) and E5 (IRS 2 and/or R 2) yielded positivity rates of 59% and 48%, respectively (table 1). Interobserver variation was low. In summary, assessment of immunoreaction by two independent investigators was

Table 2 Relationship of established prognostic factors and cathepsin D expression to disease-free survival (DFS) and overall survival (OS) for the total study group and for node negative and node positive patients (univariate analyses)

Prognostic factors		TI-4 N0-2 M0 (n = 270)		TI-4 N0 M0* (n = 120)		TI-4 N1-2 M0* (N = 145)		
		DFS	OS	DFS	OS	DFS	OS	
Age	≤ 50y v >50y	0.899	0.769	0.184	0.125	0.479	0.208	
Menopause	pre v post	0.76	0.963	0.09	0.125	0.821	0.367	
Tumour size	2 cm v 2-5 cm	0.067	0.064	0.97	0.91	0.103	0.084	
	2 cm v >5 cm	0.000009	0.000001	0.07	0.083	0.0000001	0.0000001	
	2-5 cm v >5 cm	0.000002	0.000001	0.165	0.094	0.0000001	0.0000001	
Node status*	negative v positive	0.00001	0.00004					
Tumour grade	GI v GII	0.246	0.42	0.189	0.579	0.759	0.894	
	GI v GIII	0.002	0.02	0.291	0.739	0.0042	0.019	
	GII v GIII	0.002	0.002	0.85	0.77	0.00004	0.0002	
Receptors	negative v positive	0.014	0.001	0.21	0.265	0.0002	0.0002	
Cathepsin D	Biochemistry							
	Tumour cells	negative v positive	0.122	0.183	0.076	0.118	0.610	0.371
	(E1:IRS=0:negative)	negative v positive	0.091	0.134	0.028	0.123	0.948	0.738
	Tumour cells	negative v positive	0.0022	0.0034	0.046	0.073	0.265	0.295
	(E2:IRS ≤ 1:negative)	negative v positive	0.02	0.049	0.21	0.35	0.18	0.06
	Stroma cells	negative v positive	0.625	0.084	0.056	0.177	0.678	0.477
	(E3:R ≤ 1:negative)	negative v positive	0.001	0.005	0.033	0.045	0.347	0.261
	E4	negative v positive						
	(IRS=0 and R ≤ 1:negative)	negative v positive						
	E5	negative v positive						
(IRS ≤ 1 and R ≤ 1: negative)	negative v positive							

* Axillary lymphadenectomy was performed in 265 patients. E1-E5: different types of immunohistochemical evaluation.

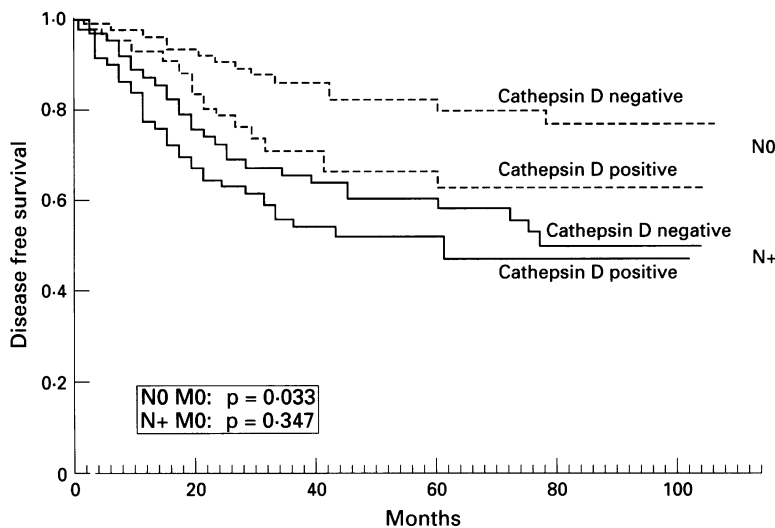


Figure 1 Kaplan-Meier curves for disease-free survival in node positive (continuous line) and node negative patients (broken line), subdivided according to immunohistochemical cathepsin D expression (evaluation formula E5).

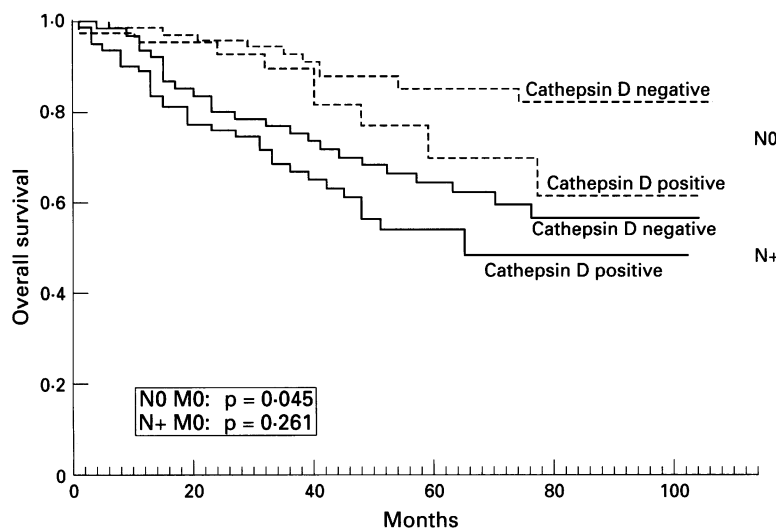


Figure 2 Kaplan-Meier curves for overall survival in node positive (continuous line) and node negative patients (broken line), subdivided according to immunohistochemical cathepsin D expression (evaluation formula E5).

identical in 92% of tumours (95% concordance for tumour cells, 89% concordance for stromal cells).

Immunoradiometric measurement of cathepsin D concentrations in tumour cytosols revealed values exceeding the cut off level (40 pmol/mg) in 57% of samples. The results of all the various immunohistochemical evaluations (E1-E5) correlated with the biochemical findings (concordance rates ranging from 64% to 73%). Correlation was highest with E5 (table 1).

Patients in our study group had been diagnosed as having breast cancer between 1983 and 1988. Follow up was complete in 262 women. Median observation time was 68 months (61-138 months). Seven women died from causes other than breast cancer, with no evidence of disease; 165 women (63%) were in complete remission; 90 women (34%) had a recurrence, and 71 of the latter group (27% of the total study group) died from their tu-

mours. In the node negative group (120 patients, complete follow up in 117 patients, median observation time 71 months), three women died from other causes with no evidence of disease, 89 (76%) had no evidence of disease, 25 (21%) had a relapse, and 18 (15%) of the latter group died from the disease. For the node positive group (145 patients, complete follow up in 141 patients, median observation time 48 months) the corresponding figures were four deaths not related to breast cancer, 75 with no evidence of disease (53%), 62 relapses (44%), and 54 tumour related deaths (38%).

Survival analyses (log-rank test using Cox-Mantel's χ^2 p value) showed tumour size and nodal status to be of high prognostic significance. In node negative patients none of the established prognostic indices bore any relation to survival rates (table 2). However, in the node positive group, tumour size, tumour grade, and steroid hormone receptor status were all significantly related to clinical outcome (table 2). With respect to immunoradiometric cathepsin D measurement, a weak correlation with prognosis (disease-free survival: $p=0.076$; overall survival: $p=0.118$) was found in node negative patients, but none at all in node positive patients (disease-free survival: $p=0.61$; overall survival: $p=0.371$). With respect to immunohistochemical cathepsin D detection, significant correlations with prognosis were found in node negative patients when evaluation E5 (disease-free survival: $p=0.033$; overall survival: $p=0.045$) was used. For node positive patients none of the immunohistochemical evaluation criteria bore any relation to prognosis ($p > 0.05$) (table 2; figs 1 and 2).

Further analysis was based on the E5 evaluation of the immunohistochemical findings, which correlated most closely with prognosis. Table 3 relates immunohistochemical expression of cathepsin D to established prognostic indices (that is, nodal status, tumour size, tumour grade, steroid receptor status, age, and menopausal status) in the total study group and in the node negative and node positive subgroups. Cathepsin D detection correlated with nodal status only ($p=0.006$). For node negative patients stepwise Cox regression showed that tumour size, tumour grade, and hormone receptor status were of prognostic significance for disease-free survival and overall survival, whereas both immunoradiometric and immunohistochemical forms of cathepsin D expression were irrelevant (table 4). For node negative patients Cox regression showed that immunohistochemically detected cathepsin D expression had prognostic value (disease-free survival: $RR=2.66$, $p=0.034$; overall survival: $RR=2.24$, $p=0.058$), while all the other indices tested were shown to be unrelated to survival (table 5).

Discussion

About 20-30% of node negative patients have a relapse and eventually die from breast cancer, whereas 70-80% survive.³⁶⁻³⁸ Choosing patients for adjuvant treatment, which has been

Table 3 Correlation between classic prognostic factors and immunohistochemical cathepsin D detection (evaluation formula E5) for the total study group and node negative and node positive sub-groups

		T1-4 N0-2 M0 (n=270)			T1-4 N0 M0* (n=120)			T1-4 N1-2 M0* (N=145)		
		Negative n	Positive n	p	Negative n	Positive n	p	Negative n	Positive n	p
Age	≤50 years	54	38		31	13		23	24	
	>50 years	87	91	0.126	44	32	0.171	42	56	0.491
Menopause	pre	46	28		25	10		21	17	
	peri	14	12		8	4		5	8	
	post	81	89	0.113	42	31	0.360	39	55	0.316
Tumour size	2cm	56	41		38	22		18	19	
	2-5cm	71	67		34	20		36	45	
	>5cm	14	21	0.192	3	3	0.810	11	16	0.817
Node status*	0 positive	75	45							
	1-3 positive	42	42							
	≥4 positive	23	38	0.006						
Tumour grade	I	24	19		14	7		9	12	
	II	74	71		36	31		38	37	
	III	43	39	0.858	25	70	0.059	18	31	0.301
Receptors	negative	45	39		25	79		20	29	
	positive	96	90	0.766	50	36	0.117	45	51	0.488

* Axillary lymphadenectomy was performed in 265 patients.

Table 4 Multivariate analysis for disease-free survival and overall survival in node positive patients (n=141). The following variables were included: cathepsin D detection by bio-chemistry (IRMA) and immunohistochemistry (IH) (evaluation formula E5), tumour size, tumour grade according to Bloom and Richardson, and steroid hormone receptor status

T1-4 N1-2 M0	Disease-free survival			Overall survival		
	p	Relative risk	(95% confidence interval)	p	Relative risk	(95% confidence interval)
Tumour size	<<0.001	1.8	(1.36-2.39)	<<0.001	2.13	(1.60-2.84)
Tumour grade	0.017	1.7	(1.10-2.53)	0.04	1.58	(1.01-2.36)
Receptors	0.04	0.6	(0.38-0.94)	0.06	0.89	(0.62-1.11)
Cathepsin D IH	0.25	1.37	(0.80-2.37)	0.20	1.45	(0.82-2.57)
Cathepsin D IRMA	0.62	1.15	(0.66-2.00)	0.88	1.05	(0.82-2.57)

Table 5 Multivariate analysis for disease-free survival and overall survival in node negative patients (n=117). The following variables were included: cathepsin D detection by bio-chemistry assay (IRMA) and immunohistochemistry measurement (IH) (evaluation formula E5), tumour size, tumour grade according to Bloom and Richardson, and steroid hormone receptor status

T1-4 N0 M0	Disease-free survival			Overall survival		
	p	Relative risk	(95% confidence interval)	p	Relative risk	(95% confidence interval)
Cathepsin D IH	0.034	2.66	(1.03-6.85)	0.058	2.24	(0.99-6.27)
Receptors	0.21	0.58	(0.24-1.37)	0.15	0.49	(0.18-1.30)
Tumour grade	0.26	1.5	(0.75-2.99)	0.24	1.34	(0.80-2.42)
Tumour size	0.37	1.3	(0.76-2.10)	0.39	0.96	(0.73-3.25)
Cathepsin D IRMA	0.52	0.8	(0.41-1.58)	0.63	0.86	(0.48-1.56)

shown to increase survival³⁹ but may cause severe side effects, is always a challenging decision whereby the risk of overtreatment must be weighed against the probable benefits to certain patients. In recent years efforts have therefore been made to search out and test potential markers of prognosis in node negative patients.^{36 37}

Numerous reports including background research^{1 3 15-18 20 40-42} and other purely clinical studies^{5-12 14 23-25 27 28 43-45} have provided evidence for a correlation between cathepsin D over-expression and an increased tendency towards invasive growth and metastasis with poor clinical outcome. These observations may be explained by the proteolytic function of cathepsin D, which is thought to facilitate tumour cell invasion by digestion of proteoglycans of the interstitial matrix and basal membrane.¹¹⁸ An

additional autocrine mitogenic effect is the hypothesised mechanism by which this protease promotes metastasis.³ High concentrations of cathepsin D have been found in breast cancer cells and macrophages, especially in their endosomes where biologically active proteins are degraded.¹⁷ Mathieu *et al*⁴¹ described an intense interaction between cathepsin D and insulin-like growth factor-II (IGF-II) through which the mitogenic effect of IGF-II is enhanced even at low concentrations of cathepsin D.

Up to now cathepsin D concentrations in tumours have been measured chiefly in cytosols,^{5-7 9 10 12 13 23 46 47} using a sandwich double determinant immunoassay (M1G8, D7E3).⁴⁸ Preparation of cytosols requires a critical amount of tumour tissue exceeding 300-500 mg. The increasing frequency of small breast carcinomas demands techniques such as

immunohistochemistry whereby predictors can be detected even in minimal amounts of tissue or in routinely fixed and processed surgical specimens. The ability to use stored specimens also allows retrospective testing of large groups of breast cancer patients. Moreover, immunohistochemistry has the major advantage of combining a topoanatomical localisation of cathepsin D with histological characterisation of reactive cells. It is therefore of considerable clinical interest to compare immunohistochemical detection methods for cathepsin D with the established immunoradiometric assay.

Garcia *et al*^{20 40} were the first to describe the immunohistochemical detection of cathepsin D in benign breast lesions and in breast carcinomas. In the meantime additional accounts of immunohistochemical cathepsin D detection in breast cancer have been published.^{4 8 11 14 22 25 27-29 43-45 49-52} It is difficult to compare these studies because there are differences with respect to nodal status, the antibodies and the form of detection used, and the method of scoring. Using different cut off values, positivity rates in tumour cells varied between 36%^{8 44} and 73%,¹⁴ or in macrophages between 35%⁸ and 89%.⁴⁵ These discrepancies may be explained, at least in part, by the use of different antibodies directed against different epitopes and by the heterogeneity of the study groups. They show the importance of devising a commonly accepted method of scoring.

A few studies have compared immunohistochemical and immunoradiometric methods of cathepsin D determination and reported a good correlation,^{11 22 25-28} results that are confirmed by our data. However, these investigators did not discriminate between the two methods as to prognostic value and only one report²⁵ was based on more than 100 tumour specimens.

The cathepsin D staining pattern in tumour cells, and "tumour infiltrating macrophages", which resembles the lysosomal localisation of the antigen and has also been described in other reports.^{4 8 11 14 40 43 44 49 50 52} In some neoplasias the presence of tumour associated macrophages correlates with tumour invasion, tumour grade, and necrosis.⁵³ In agreement with other study groups,^{8 11 25 28 45} we found a strong coexpression of cathepsin D in tumour cells and in stroma. The evaluation of positive tumour cells (E1, E2) is straightforward, whereas the assessment of tumour associated macrophages (E3) is harder, because a definitive histological classification of the latter can be difficult. This is reflected by the somewhat lower interobserver concordance for E3. Winstanley *et al*¹⁴ based their evaluation of cathepsin D overexpression on positive tumour cells only, and reported a comparably good interobserver concordance (90%). The agreement between the assessments of different research assistants in Stonelake's group¹¹ was also high at 91%. In order to discriminate between varying degrees of antigen expression, staining intensity of tumour cells is recorded in the form of an immunoreactive score. We did not employ this score in tumour associated stroma because the

distribution and staining intensity of positive macrophages varied considerably. Both tumour cells and macrophages should be evaluated as long as the function of cathepsin D positive macrophages in tumour neogenesis and tumour spread remains unclear and staining of stromal cells correlates with survival. Their combined evaluation (tumour cells or macrophages or both) according to formula E5, by which weak staining in sporadic tumor cells (IRS 1) and immunoreaction in single stromal cells (R 1) is recorded as negative, yielded the best correlation to IRMA and, more importantly, information on the clinical course of the disease.

Subjective error in the assessment of immunohistochemical staining is a major concern. Our group E 5 allowed clear characterisation of the majority (79%) of tumours. In 32% of tumours a distinct absence of specific staining made a negative scoring unequivocal. On the other hand 47% of tumours shown a clear and immediately obvious positive immunoreaction. Cases with exclusively low immunoreaction in stromal cells (9%) or with a tumour cell IRS 1 (12%) are dubious and require more careful consideration.

When cathepsin D detection was compared with case history and tumour characteristics, we found no correlation between cathepsin D and age, menopausal status, or receptor status, thus confirming the results of other investigators.^{4 8 43 44 50} Like three other groups,^{14 27 45} we found the relation between cathepsin D expression and nodal status to be highly significant, but in contrast to Isola *et al*⁸ or Winstanley *et al*¹⁴ we did not find any correlation between cathepsin D expression and tumour size.

With respect to the prognostic value of immunohistochemical cathepsin D detection, the published results are equally confusing. In contrast to our findings, Têtu *et al*⁴⁴ reported a significant correlation between disease-free survival of node positive patients and positive immunoreaction in stromal cells, from which they concluded that macrophages played a significant role in invasive tumour growth and metastasis.⁴⁴ Kandalaf *et al*⁵⁰ found no correlation between cathepsin D detection and survival in either node negative or node positive patients. Henry *et al*⁴³ reported a significant relation between cathepsin D expression and disease-free survival in oestrogen receptor positive, node negative cases. Winstanley *et al*¹⁴ also showed a significant correlation between cathepsin D detection and overall survival, but only after univariate analysis. However, in their evaluations these investigators took no account of nodal status. In their study of stage I breast cancer patients, Armas *et al*⁴ also used univariate tests but found that cathepsin D expression was related to neither disease-free survival nor overall survival. Using univariate analysis Eng-Tan *et al*²⁸ showed a correlation between immunohistochemically detected cathepsin D and disease-free survival but not overall survival in breast cancer patients; however, they failed to establish it as an independent prognostic factor. Isola *et al*⁸ detected strong correlations between cathepsin D expression

and disease-free and overall survival in node negative patients using both univariate and multivariate analyses. In a group of 86 stage-heterogeneous breast carcinomas Visscher *et al.*⁴⁵ found that neither stromal nor tumour cell positivity alone correlated with recurrence-free survival; however, additive staining in both compartments was strongly predictive in all patients and in the node positive subgroup. We confirmed these latter results. In an earlier report Tandon *et al.*¹² showed a significant correlations between cathepsin D expression and prognosis in node negative patients using univariate and multivariate analyses. However, recently this group⁵⁴ failed to reproduce their findings when using western blot analysis and immunohistochemistry on cell pellets.

We present data that support the suggestion that immunohistochemical detection of cathepsin D could be used to identify patients with poor prognosis in the important group of node negative breast cancer patients. We have shown that the criteria on which the evaluation of immunohistochemical staining is based are most critical for a relevant linkage of immunohistochemical findings to clinical outcome. Our data should now be supplemented by prospective immunohistochemical studies with well defined and uniform criteria for evaluating immunoreactivity. To this end the scoring system presented in this report may be of value.

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