Cathepsin D detected by automated and quantitative immunohistochemistry in breast carcinomas: correlation with overall and disease free survival

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Abstract

Aim—To determine the prognostic significance and clinical relevance of cathepsin D detected by immunocytochemical assays (ICAs) in breast carcinomas.

Methods—151 patients presenting with palpable or impalpable breast carcinomas and who had not received any kind of adjuvant chemotherapy or endocrine therapy who were operated from January 1986 to May 1987 were studied. ICAs of tumour specimens were performed in optimal technical conditions (frozen sections, automated immunoperoxidase technique (Ventana), and computer assisted analysis of digitised coloured microscopic images (SAMBA)) to determine cathepsin D immunocytochemical expression. Results of quantitative ICAs were correlated with overall and disease free survival over 8.4 years of follow up in axillary lymph node positive and negative patients.

Results—Cathepsin D immunocytochemical expression in tumours of 15% or more was significantly associated with poor overall survival in the whole group and in node positive patients (Kaplan Meier, log rank test $p = 0.003$ and $p = 0.007$); however, it was not correlated with survival in node negative patients. Cathepsin D immunocytochemical expression ($> 15\%$) correlated with short disease free ($p = 0.015$) and short recurrence free survival ($p = 0.021$) in the group as a whole but not when node positive and negative patients were evaluated separately.

Conclusions—In optimal conditions (automated and quantitative ICAs on frozen sections) cathepsin D immunohistochemical expression is a significant prognostic indicator in terms of overall, disease free, and recurrence free survival; however, there is no correlation when node negative patients are considered separately.

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Keywords: automated immunoperoxidase; image analysis; immunocytochemistry; cathepsin D; breast carcinomas; survival

Cathepsin D is a lysosomal acidic protease regulated by oestrogens in the MCF-7 breast

cancer cell line.¹ Experimental studies of cathepsin D have shown proteolytic activity associated with mitogenic activity,² suggesting a potential role for cathepsin D in extracellular matrix degradation and tumour progression³ in human breast carcinomas.

The prognostic value of cathepsin D has been evaluated in several large series of breast carcinomas. These studies have shown that overexpression of cathepsin D in breast carcinomas increases the probability of recurrence and of poorer survival.⁴⁻⁸ More recent immunohistochemical studies have also demonstrated the prognostic significance of cathepsin D expression in breast carcinomas⁹⁻¹⁵ with significant correlation between cathepsin D expression and either the patients' outcome or other major prognostic factors.^{14, 15}

In a previous study¹⁶ we showed that cathepsin D immunolabelling in tissue sections may vary with the tissue fixation method and paraffin wax embedding, and that it relies on the accuracy of the method used to evaluate the immunoprecipitates on tissue sections. Also, cathepsin D immunoreactivity may vary significantly depending on the antibody used.^{15, 17} It is likely that heterogeneous data reported concerning the prognostic significance of cathepsin D expression may result, at least partly, from the lack of standardisation of immunohistochemical technique and from technical bias.

In the present study, we attempted to clarify the clinical relevance of cathepsin D immunocytochemical assays (ICAs) using optimal technical conditions including frozen tissue samples, an automated (Ventana medical system) immunohistochemical procedure, and computer assisted analysis of digitised microscopic images (SAMBA image analyser) to evaluate positive immunoreactions. The results were correlated with the patients' outcome over 8.4 years of follow up.

Materials and methods

One hundred and fifty one patients presenting with palpable or impalpable breast carcinomas and who had not received any kind of adjuvant chemotherapy or endocrine therapy who were operated from January 1986 to May 1987 were studied. Follow up ranged from nine months to 101 months (mean (SD) 61.5 (25), median 64.9).

The histological examination of the surgical specimens was performed on paraffin wax

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Figure 1 Immunostaining of frozen sections with anti-cathepsin D (MIG8 monoclonal antibody, Ventana device) in ductal carcinoma of non-special type, grade II. (Left) Mild staining; (Right) Pronounced staining.

embedded sections stained with haematoxylin and eosin, and saffronin.

IMMUNOSTAINING AND IMAGE ANALYSIS

Tissue

Tissue fragments were sampled for immunostaining by the pathologist immediately after the intraoperative diagnosis using frozen sections. The size of the fragments varied according to the tumour size (an average of $5 \times 4 \times 2$ mm). The frozen fragments were sampled in dense tumour areas, lacking in grossly visible adipose tissue, promptly dipped in liquid nitrogen and stored at -80°C in the laboratory tumour library (established for prospective studies in 1986). Immunodetections were each performed on $5\text{ }\mu\text{m}$ thick sections (cryostat Leica 3000, Rueil Malmaison, France).

Immunoperoxidase

Monoclonal (MIG8) mouse antihuman cathepsin D¹⁹ was purchased from CisBio (Compiègne, France). The immunoperoxidase technique was performed using an automatic

immunostaining device (Ventana, Medical System, Strasbourg, France) and Ventana kits.²⁰⁻²²

Image analysis

The immunoprecipitates were analysed using an Axiophot microscope (Zeiss) and a 3 CCD Sony Camera and then processed in an image analysis device (SAMBA 2005, Alcatel TITN, Grenoble, France).¹⁶⁻²³ The two parameters of the densitometric analysis, the percentages of immunostained surfaces (versus counterstained surface) and mean optical density (MOD), which depends upon the staining intensity (SAMBA arbitrary units scale, 0-255), were obtained as reported previously.¹⁶⁻²⁴

STATISTICAL ANALYSIS

Analysis of disease free survival and overall survival was performed using the Kaplan Meier method. The difference between curves was evaluated with the Mantel Cox test (or log rank test) for censored survival or events observations. All computations were done with BMDP statistical software (University of California, Berkeley, California, USA). The percentages of cathepsin D stained surface evaluated by image processing device were stratified and correlated with major events during the course of the disease (distant metastases and local recurrences) and with overall survival, to define immunohistochemical thresholds of prognostic significance. An optimal cathepsin D cut off point of positive surface endowed with prognostic significance was determined after statistical validation.²⁵

Results

PATIENTS

The patients' ages ranged from 34 to 79 years (mean 53.2 (12.3)). Tumour size ranged from 4 to 51 mm (mean 22.5 (18.5)). At 101 months (8.4 years), the records showed that 28 of the 151 patients (18.5%) had died, 21 (14%) had developed distant metastases, and 16 (10.5%) had local recurrence.

TUMOURS

All the tumours were invasive carcinomas. The majority (98 of 151, 65%) was ductal type, and the remainder were lobular type (38 of 151,

Figure 2 Distribution of cathepsin D immunostained surface (Cath surface) in 151 breast carcinomas evaluated by automated (Ventana) immunoperoxidase on frozen sections; quantitation by computer assisted analysis (SAMBA) of digitised coloured microscopic images (ordinate, percentage of patients; abscissa, percentage of immunostained surface versus counterstained surfaces).

Figure 3 Overall survival of patients ($n = 151$) correlated with cathepsin D immunostained tumour surface (Cath) (mean surface) evaluated by computer assisted image analysis of digitised microscopic images (left) in entire series and (right) in node positive patients (Kaplan Meier, log rank test).

25%) or of various histological types including mucinous, medullary, papillary, apocrine or cribriform, or mixed types (15 of 151, 10%). Ductal invasive carcinoma grade 1¹⁸ accounted for 16% of the tumours, grade 2 for 59%, and grade 3 for 25%. Axillary lymph node dissection was carried out in 142 patients. The mean (SD) number of nodes found in axillary resection was 14.2 (5). The histological examination showed that 69 of 142 (48.6%) patients were node positive and 73 (52.4%) node negative. Twenty one of the node positive patients had more than three metastatic lymph nodes.

CATHEPSIN D DISTRIBUTION IN TISSUE SECTIONS

Cathepsin D immunohistochemical expression was similar to that observed previously in breast tumours¹⁶ with positive staining predominantly seen in epithelial cells, and was heterogeneous on tissue sections. All tumours were cathepsin D positive but the immunopre-

cipitate distribution was variable. Some tumours were markedly positive and others faintly positive (fig 1). However, the heterogeneity of cathepsin D expression essentially concerned the surfaces of immunostaining whereas the intensity of staining was most often homogeneous and strong.

The variations in the cathepsin D positive surfaces on tissue sections are shown fig 2. Cathepsin D surface in epithelial cells evaluated by computer assisted image analysis varied from 1% to 60% (mean 18.1 (13.7)). The mean optical densities reflecting the intensity of staining varied from 75 to 89 arbitrary units (mean 82 (27)). As the staining intensity was not significantly variable in the series investigated, this parameter was not used for the correlation studies with follow up. Thus the QIC (quantitative immunocytochemical) index including the staining intensity²⁶ was not useful and not worthy of statistical analysis.

Table 1 Overall survival of patients (8.4 year follow up) correlated with mean cathepsin D immunostained surfaces evaluated by computer assisted analysis of digitised microscopic images

	Cathepsin D immunostained surface	
	≤15%	>15%
All patients (n=151)		
Alive	77/123	46/123
Dead	9/28	19/28
Node negative patients (n=73)		
Alive	45/65	20/65
Dead	5/8	3/8
Node positive patients (n=69)		
Alive	30/52	22/52
Dead	3/17	14/17

Table 2 Disease free survival of patients (8.4 year follow up) correlated with mean cathepsin D immunostained surfaces evaluated by computer assisted analysis of digitised microscopic images

	Cathepsin D immunostained surface	
	≤15%	>15%
All patients (n=151)		
No disease	69/114	41/114
Disease	17/37	24/37
Node negative patients (n=73)		
No disease	40/57	17/57
Disease	10/16	6/16
Node positive patients (n=69)		
No disease	27/49	22/49
Disease	6/20	14/20

CATHEPSIN D IMMUNOHISTOCHEMICAL EXPRESSION AND SURVIVAL

Cathepsin D immunostained surfaces (cut off point 15%) correlated significantly ($p = 0.003$) with overall survival (fig 3). Patients with tumours with a large cathepsin D positive surface had poorer survival than those with tumours with a small cathepsin D positive surface (table 1). However, when the whole series of patients ($n = 151$) was stratified into node positive ($n = 69$) and node negative patients ($n = 73$), the cathepsin D ($> 15\%$) prognostic significance was only retained ($p = 0.007$) in node positive patients (fig 3) and not in node negative ($p = 0.63$).

Cathepsin D correlated with ($p = 0.015$) disease free survival in the overall patient group (fig 4) but not when node positive ($p = 0.07$) and node negative patients ($p = 0.45$) were evaluated separately (table 2).

Large cathepsin D positive surfaces also correlated ($p = 0.021$) with recurrence free survival in the group as a whole (fig 5, table 3) but not when node positive and node negative patients were evaluated separately ($p = 0.18$, $p = 0.08$, respectively; not shown).

Cathepsin D immunoreactions did not correlate with metastasis free survival in the group as a whole ($p = 0.24$) (table 4) or in the

Table 3 Recurrence free survival of patients (8.4 year follow up) correlated with mean cathepsin D immunostained surfaces evaluated by computer assisted analysis of digitised microscopic images

	Cathepsin D immunostained surface	
	≤15%	>15%
All patients (n=151)		
No recurrence	81/135	54/135
Recurrence	5/16	11/16
Node negative patients (n=73)		
No recurrence	46/64	18/64
Recurrence	4/9	5/9
Node positive patients (n=69)		
No recurrence	32/64	32/64
Recurrence	1/5	4/5

Table 4 Metastases free survival of patients (8.4 year follow up) correlated with mean cathepsin D immunostained surfaces evaluated by computer assisted analysis of digitised microscopic images

	Cathepsin D immunostained surface	
	≤15%	>15%
All patients (n=151)		
No metastases	76/130	54/130
Metastases	10/21	11/21
Node negative patients (n=73)		
No metastases	46/67	21/67
Metastases	4/6	2/6
Node positive patients (n=69)		
No metastases	28/56	28/56
Metastases	5/13	8/13

node negative (p = 0.81) and node positive subgroups (p = 0.44) (not shown).

Discussion

Node negative cancers account for the large majority of all breast cancers.²⁷ Not all patients with node negative cancers have to be treated with additional systemic therapy as approximately 70% are cured without adjuvant therapy and because the benefits of currently available regimens are small.²⁷ Therefore, in this group of patients the use of prognostic factors may be particularly relevant for selecting patients with a high risk of recurrence who should benefit from adjuvant therapy. The overexpression of cathepsin D has been reported to be such a prognostic indicator⁴⁻¹² of strong relative power, as shown by multivariate analysis expressed by rank-order chart of prog-

nostic factors. These results may justify the routine evaluation of cathepsin D in patients with breast cancers, provided that the technical means of measurements are standardised.

Recent reports^{9 26 28} have demonstrated a good correlation between cathepsin D immunoassays in cytosol and ICAs. Although immunohistochemical techniques are often considered as a qualitative unreliable method of detection they have the advantage of requiring a minimal amount of tissue.²⁹ Moreover, ICAs document more accurately the distribution of antigens within tumours.^{12 16 28}

Some immunohistochemical studies of cathepsin D have been reported in the recent literature.^{1 9-14 26 28-32} Some of these studies showed results concordant with those of others using different methods for cathepsin D evaluation in tissue.⁴⁻⁸ However, other reports were in disagreement showing that cathepsin D expression in tissue sections was not an independent prognostic indicator.^{14 30-32} The differences in the procedures used to detect cathepsin D in tissue may be responsible for these discrepancies. In particular, in a previous study¹⁶ we showed that fixation and paraffin wax embedding appeared to decrease significantly (at least 40%) cathepsin D antigenicity. It is also probable that semiquantitative methods used to evaluate the immunoprecipitates produce another type of analytical bias as they rely on observers' subjectivity. In contrast, digital image analysis, as for other markers,²⁴ provides a standardised means of immunoprecipitates evaluation. It has recently been used for cathepsin D immunodetections,^{16 26 28} particularly to determine the cut off point of prognostic significance.

The polyclonal or monoclonal antibodies used may also produce for variable cathepsin D immunolabelling.¹⁴ Similarly manual performance of immunocytochemical tests may be responsible for the lack of reproducibility of antigen immunodetections on tissue sections. Automation of immunocytochemical assays using a specific device permits the running of large batches of slides in similar conditions.

In conclusion, in optimal technical conditions using automated and quantitative ICAs on frozen sections, we were able to show that

Figure 4 Disease free survival of all patients (n = 151) correlated with cathepsin D immunostained surface (Cath) (mean surface) evaluated by computer assisted analysis (SAMBA) of digitised coloured microscopic images (ordinate, percentage of patients; abscissa, percentage of immunostained surface versus counterstained surface).

Figure 5 Recurrence free survival of all patients (n = 151) correlated with cathepsin D immunostained surface evaluated by computer assisted analysis of digitised coloured microscopic images (ordinate, percentage of patients; abscissa, percentage of immunostained surface versus counterstained surface).

marked cathepsin D expression (cut off point 15%) in breast carcinomas was significantly associated with overall poor survival and short disease free survival but not in node negative patients. Thus marked cathepsin D expression in tissue has some clinical relevance but limited prognostic significance in early breast carcinomas.

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