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The Expression of Lysosomal Proteinases and Their Inhibitors in Breast Cancer: Possible Relationship to Prognosis of the Disease

Tamara T LAH,[1,2] Janko KOS,[2] Andrej BLEJEC,[1] Snežana FRKOVIČ-GEORGIO,[3] Rastko GOLOUH,[3] Ivan VRHOVEC,[3] and Vito TURK[2]

[1]Laboratory of Molecular Biology and Biochemistry, National Institute of Biology; [2]Department of Biochemistry and Molecular Biology, Jozef Stefan Institute, and KRKA d.d. Ljubljana; [3]Departments of Pathology and Clinical Biochemistry, Institute of Oncology; Ljubljana, Slovenia

Proteolytic enzymes have been proposed as new biological prognostic indicators to facilitate decisions about treatment of breast cancer patients following surgery. We reported earlier that the activities of cysteine proteinases (CP), cathepsin (Cat) B and cathepsin (Cat) L and the expression of stefin A might be associated with breast tumor progression and prognosis. Here, the protein concentrations of Cats D, B and L and stefin A have been measured in a series of 60 matched pairs of breast tumours and control adjacent tissues, using ELISAs developed in our laboratory. Median tumor concentrations of Cat D (47 pm/mg), Cat B (222 ng/mg) and Cat L (88 ng/mg) were significantly ($p<0.0005$) increased by 7 fold, 27 fold and 6 fold, respectively. Much greater increases in the activities of Cat B (63 fold) and of Cat L (274 fold) were found, indicating enhanced activation of cysteine proteinases in tumors, due either to proteolytic activation of proCat B and proCat L and/or to a decrease in specific endogenous cystatins. However, the 1.6-fold decreased ($p<0.0001$) levels of inhibition by cystatins could not be entirely responsible for more than 100-fold increased ratio of CP:cystatins activity. Moreover, stefin A was either increased or decreased in tumor samples, resulting in a 1.4-fold median increase in tumors. Comparing the biological parameters with the established histo-pathological prognosticators, we found that the increased protein concentration of Cat B was associated with lymph node involvement ($p<0.009$) and higher stage ($p<0.003$), and both Cat B and Cat L activities were more increased in high grade tumours ($p<0.05$). Survival analysis revealed that stefin A was the most significant prognostic factor for disease-free ($p<0.008$) and overall survival ($p<0.02$), followed by increased Cat B activity and protein concentration. Cat L was of borderline significance while Cat D was not significant for prognosis. We conclude that enhanced activation of CP, due partially to an imbalance between cysteine proteinases and inhibitors is linked to the progression of breast cancer. Larger sample size is needed to confirm the prognostic significance of stefin A, Cat B and Cat L.
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Key words: breast cancer, cathepsin B, cathepsin D, cathepsin L, lysosomal enzymes, metastasis, prognostic factors, stefins

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Correspondence: Tamara LAH, National Institute of Biology, Večna pot 111, 1000 Ljubljana Tel: 386-61-123-3388; Fax: 386-61-123-5038; E-mail: tamara.lah@ijs.si

Abbreviations: AMC, 4-methoxy-7 amino coumarine; E-64, L-epoxy succinylleucylamido(4-guanidino) butane Cat, Cathepsin; CP, cysteine proteinase; CPI, CP inhibitor; DFS, disease-free survival; ELISA, enzyme linked immunosorbent assay; ER, estrogen receptor; OS, overall survival; PA, plasminogen activator; PR, progesterone receptor; u-PA, urokinase type of plasminogen activator; u-PAR, plasminogen activator receptor, Z-, benzoxycarbonyl.

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Introduction

Proteinases and their endogenous inhibitors, including cathepsin (Cat) D,[32,33] urokinase (u-PA),[10] plasminogen activator inhibitor (PAI-I),[19] and a variety of metalloproteinases,[9] play an important role in tumor progression. A large body of literature has accumulated,[8,38] demonstrating that lysosomal cysteine proteinases and their endogenous inhibitors[14] are also actively involved in the process of cancer invasion and metastasis. All these components of proteolytic systems, possibly acting independently and/or in a proteolytic cascade in tumor and/or stromal cells during various steps of tumor metastasis, are also potential candidates for prognostic markers.[16]

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Cat D was the first lysosomal enzyme to be studied in breast cancer patients and in a number of clinical studies, Cat D concentration in breast tumors was found to be an independent prognostic parameter associated with occurrence of clinical metastasis and shorter survival.[33] Cysteine proteinase Cat B was first associated with breast carcinoma by Poole et al,[29] while Cat L was found to be upregulated at the mRNA level in many types of human tumors, including breast carcinoma.[7] In the first pilot study of cysteine proteinases (CPs) in matched pairs of breast and control tissues, we have demonstrated much greater increases in the activities of Cat B and Cat L than of Cat D activity.[26] We have also suggested that Cat L levels might be of importance for prognosis of disease-free and overall survival. This was confirmed by Thomssen et al[41] demonstrating high impact of Cat L and lower significance of Cat B protein for prognosis of disease-free (DFS) and overall survival (OS).

CPs are regulated by a variety of endogenous inhibitors (CPIs), comprising the superfamily of cystatins. Variations of cystatin levels in tumors have been reviewed.[8,38] The imbalance between CP and cystatins seems to be associated with an increase in metastatic potential in some experimental tumors,[6] in lung carcinoma[12,21] and in amelanotic melanoma.[39]

We reported a decrease in total cystatin activities in tumors of about two thirds of breast cancer patients who had been identified by histopathological and clinical prognostic factors for bad prognosis.[26] In some tumors, stefin A was downregulated at both mRNA and protein levels and we speculated that the stefin A gene belongs to the family of genes, suppressed in tumors, as was also suggested by studies on skin carcinoma[17] and prostate adenocarcinoma.[36] Due to the variety of possible CP and cystatin interactions, these proteins should be determined separately at the levels of mRNA, protein and activity, in order to understand the balance between these two groups of antagonistic molecules in tumor progression.

In this study, we have measured the activities of Cat B, Cat L and cystatins, as well as the protein concentrations of Cat D, Cat B, Cat L and stefin A in the cytosols of 60 matched pairs of tumor and control adjacent tissues from breast carcinomas. The aim of the study was first to investigate possible co-ordination in the up-regulation of three lysosomal proteinases, Cat D, Cat B and Cat L at both activity and protein levels. Secondly, we aimed to confirm the previously observed imbalance between CP and cystatins, particularly stefin A, in tumors as compared to controls. Finally, we have correlated these biochemical variables with the established clinical and histopathological indicators of the progression of the disease and to the disease-free and overall survival of breast cancer patients.

Material and Methods

Patient selection and preparation of tissues

Patients diagnosed for invasive carcinoma of the breast and treated by partial or total mastectomy were selected randomly for this study. Tumors were graded according to Bloom-Richardson Elston grading system,[13] the grade for individual tumor was obtained by summing the scores for tubule formation, nuclear pleomorphism and mitotic count, each of which was given 1, 2 or 3 points. The regional lymph node involvement was determined histologically. Together with tumor size and distant metastasis, the tumors were grouped in stages I, IIA and IIB and III (A and B) according to WHO Histological Classification of Breast Tumors (Geneva, Switzerland, Ed2, 1981).

On removal, tumors and control tissues were frozen and stored at -70℃. Frozen specimens were pulverised using a micro-dismembrator for 60 sec at maximum power. The frozen powder was suspended in EORTC recommended buffer, *i.e.* 10 mM K_2HPO_4/KH_2PO_4, 1.5 mM dipotassium EDTA, 1 mM monothioglycerol, 10% glycerol, pH 7.4. The homogenates were centrifuged at 13000g for 10 min in a Sorvall RC2 refrigerated centrifuge. Supernatants, called "cytosols", were aliquoted (100 µl) and stored at -20℃ for several years before the measurements of cathepsins and stefins were performed. 60 matched pairs of tumor and adjacent non-involved tissue, which was carefully analysed by a pathologist, were included in the study.

Materials

The chemicals used were of analytical grade and were purchased from Sigma (Poole, UK). Papain (2x crystallised, type IV) and E-64 were purchased also from Sigma. Peptide substrates and inhibitors were obtained from Bachem Biochemica Gmbh (Heidelberg, Germany).

Cathepsins – Activity assays

Cathepsin B – Cat B activity was determined using modified method of Barret and Kirschke[2] as described previously.[26] Briefly, 20 µl of the supernatant was added to 280 µl of the 0.4 M phosphate buffer, pH 6.0, containing 4 mM EDTA/Na_2 and 1.2 mM dithiothreitol and incubated for 10 min at 37℃. 100 µl of the substrate Z-Arg-Arg-AMC was added to a final concentration of 60 µM and further incubated for 150 min. The reaction was terminated by the addition of 500 µl of 1 mM ice-cold iodoacetic acid. In the blank assays, running in parallel, the sample solution was replaced by the addition of bidistilled water, while the control assays contained 1 µM final concentration of E-64. Fluorescence of the product 7-AMC was read at an excitation wavelength of 370 nm and emission wave-

length of 460 nm using Perkin Elmer spectrophotometer LS5 (Norwalk, CT, USA). One enzyme unit (EU) represented the amount of the enzyme releasing one micromole of 7-AMC per ml. Specific activity was expressed in EU per g total protein in the sample. When enzyme activity was compared to immunochemically determined Cat B, the term "normalised specific activity" was used.

Cathepsin L – Cat L activity was measured using Z-Phe-Arg-AMC as a substrate. To discriminate between Cat B and Cat L activities, we measured the hydrolysis of Z-Phe-Arg-AMC in the presence and absence of 0.5 μM final concentration of Z-Phe-Phe-CHN_2, the latter inhibiting Cat L but not Cat B as described previously.[26] In brief, 100 μl of the sample was pre-incubated for 30 min at 37℃ in the activation buffer, i.e. 0.34 M acetate buffer, pH 4.2, containing 2 mM dithiothreitol and 1 mM EDTA/Na_2. After the addition of the substrate, 100 μl of Z-Phe-Arg-AMC (final concentration 100 mM) further incubations were carried out in the presence and absence of 100 μl Z-Phe-Phe-CHN_2 for 180 min. The reaction was terminated by the addition of 500 μl of ice cold 1 mM iodoacetic acid. In the blank assay running in parallel, the sample solution was replaced by bidistilled water. Enzyme units were calculated as described above for Cat B, after correcting for blanks and controls and the specific activity was expressed in EU/g protein in the sample. When specific activity was compared to immunochemically determined Cat L protein, the term "normalised specific activity" was used.

Total inhibitory activity of CPIs

The activities of cystatins, measured as the total cysteine proteinase inhibitory (CPI) activity against papain, were determined after heat treatment of the cytosols. Only in 43 patients sufficient volume of the cytosol was available to perform papain titration. Papain was additionally purified as described[20] and the inhibitory activity was referred to as the total cystatins activity.[26] Briefly, the cytosols were incubated for 10 min at 100℃ and after cooling and centrifugation 10–100 μl of cytosols were added to 100 μl of 4 nM papain in the presence of 1.3 mM EDTA, 2.5 mM dithiothreitol and 0.005% Brij 35 in 0.34 M acetate buffer, pH 5.5. After the addition of Z-Phe-Arg-AMC (5 μM final concentration), the reaction was terminated by the addition of 400 μl of 1 mM iodoacetic acid. Fluorescence was read against blank which was prepared by adding iodoacetic acid before other reagents. Controls for papain activity were prepared with buffer added instead of the inhibitors. One inhibitory unit (IU) equals one enzyme unit (EU) and represents the amount of inhibitor that totally inhibited papain activity in the assay: this was determined by extrapolation of the titration curves to zero papain activity. Specific inhibitory activity was expressed in IU/g protein in the sample.

Determination of steroid hormone receptors and protein concentration

Estrogen receptors (ER) and progesterone receptors (PR) were measured according to the protocol of BYK-Diagnostica, using dextran-assay with radiolabeled ligands. Tumors with receptor concentrations above 10 fmol/mg protein were considered receptor positive. Protein was determined with Bio-Rad protein assay,[3] using bovine serum albumin as standard protein.

Enzyme immunosorbent assay (ELISA).

ELISAs for Cat B, Cat L and stefin A were developed in our laboratory and are commercially available from KRKA d.d. (Novo mesto, Slovenia). The assays were carried out according to the manufacturer's protocol. Purified native Cat B and Cat L and recombinant stefin A were used as antigens and standards. The characteristics of the assays, linearity and precision controls were reported previously.[22]

Cathepsin B – For Cat B, immunoselective sheep and rabbit polyclonal Abs (IgG) were used as capture and detection Abs, respectively. Tumor samples in 1:100 dilution and control samples in 1:10 were added to microtiter plate wells and the assay was carried out as described.[22] As internal controls, two pools of normal breast cytosols and tumor cytosols were prepared, aliquoted (50 μl) and frozen at -70℃. Aliquots were thawed out and assayed along with the samples.

Cathepsin L – For Cat L sandwich ELISA sheep anti-Cat L immunoselective IgG was used as a capture Ab and as horseradish-peroxidase-conjugated Ab for detection. Tumor tissue samples were diluted 1:5 and control samples were diluted 1:2. According to the manufacturer, the ELISA assay for Cat L and Cat B showed no crossreactivity but recognised all molecular forms of the enzymes including the complexes with cystatins.

Cathepsin D – Commercially available solid phase two-site immunoradiometric assay (ELISA-CATH-D, CIS Bio International, Gif-sur-Yvette, France) was used and the assays were carried out according to the manufacturer's protocol. The assay detects precursor and mature forms of Cat D. Tumor tissue samples in 1:80 dilution and normal tissue samples in 1:40 dilution were used.

Stefin A – For stefin A, monoclonal Abs were used in sandwich ELISAs, C5/2 MoAb as capture and A2/2 MoAb as detection Ab, the latter being conjugated with horseradish peroxidase. Tumor and control samples were used in 1:100 dilution.

Statistical Analysis

For statistical analysis of the differences in the levels of the variables in matched breast tissue pairs (tumor and control), we used Wilcoxon's rank test and Kruskal-Wallis

test. The levels of significance were determined by Kruskal-Wallis one way ANOVA, where the probability of the differences in the medians of less than 0.05 was considered significant. The correlation between various biochemical parameters was calculated by Spearman rank test. To determine the differences in the survival probability between the groups of patients, separated either by discrete values of covariates or by optimised cut-off values of the continuous variables, we have used Kaplan–Meier and Cox univariate analysis. The cut-off values were optimised according to Abel et al.[1] by our own program (S-PLUS statistical package.)

***Table 1.* Characteristics and distribution of patients with breast carcinoma**

Patients (female) Total	*Number of patients (60)*	*Relative percent (100%)*
Invasive intraductal (IDC)	51	85
Invasive lobular (ILC)	5	8
Medullar (MED)	1	2
Mixed ductal and lobular (IDC/ILC)	3	5
Median age (years)	59 (47–72)	
Tumor size (n =41)		
– 2.0 cm (T1)	4	10
2.0 – 5.0 cm (T2)	34	83
> 5.1 cm (T3)	3	7
Lymph nodes (n=57)		
negative : 0	27	47
positive: 0–3	11	19
positive > 3	19	33
positive/negative (+)/(–)	30/27	
Stage TNM (n=57)		
I	3	5
IIA	22	39
IIB	30	53
III (A and B)	2	3
Histological grade (n=50)		
grade 1	5	10
grade 2	19	38
grade 3	26	52
Estrogen receptor (ER) status		
> 10 fm/mg (+)	27	52
< 10 fm/mg (–)	25	48
Follow-up		
No evidence of disease, NED	30	52
Alive with disease, AWD	9	16
Dead of disease : DOD	14	24
Dead of other cause (DEAD)	2	3
Lost from the follow-up	3	5
Disease-free survival (months)*	41 (16–63)	
Overall survival (months)	61 (30–64)	

Results

Patients

The characteristics of the patients selected for this study are presented in *Table 1*. 98 % of patients were identified with invasive intraductal carcinoma and lobular or miscellaneous carcinoma while one patient was diagnosed for medullary carcinoma. The population used for this study comprised patients with advanced tumors. The majority of tumors were between 2 and 5 cm diameter, half of tumors had already metastasised to the axilliary lymph nodes and consequently 53 % of patients were staged IIB. More than half of the tumors were poorly differentiated and about half of them did not express estrogen receptor (ER). Only 52 % of patients were without evidence of the disease at median disease-free follow-up, which was at 41 months. The median overall survival was 61 months. Patients did not get pre-operative radio- or chemotherapy. After removal of the primary tumor, patients were treated with chemo, hormone or radio therapy based on individual patient and not exclusively on the lymph node status.

Cathepsins and cystatins in tumor and control tissues

Cathepsins

Table 2. presents median levels of cathepsins and cysteine proteinase inhibitors, cystatins, in tumor and control tissues of the same breast. For all cathepsins tested, highly significant ($p < 0.0005$) increases in activity and protein concentration were found in tumors. We also calculated relative increase (or decrease), i.e. the normalised difference between tumor and control cytosols (T-C/C) in specific activity (RISA) and the relative increase (or decrease) in concentration (RIC) of the biochemical parameters, as reported previously.[26,27] The median values of RISA and RIC were similar to the median values of ratios between tumor and control values (Tm/Cm) as shown in last two columns of *Table 2*. Therefore, these ratios were used for further determinations of correlation and for survival analysis *(Tables 3 and 4)*. As indicated in *Table 2.*, the activities and the concentrations of Cat B and of Cat L were not increased proportionally: tumor Cat B activity (CatBa) was elevated about twice as much as the respective protein (63 fold *vs* 27 fold increase), while Cat L activity (CatLa) was elevated about forty-six times compared to its protein concentration (274-fold *vs* 5.9-fold increase). We expressed the ratio between specific Cat B

Table 2. **Cathepsins and cysteine proteinase inhibitors in tumor and control breast tissues**

Patients (n= 60)	*Control* Median (25–75 percentile)	*Tumor* Median (25–75 percentile)	*Significance*[a] p	*RISA*[b] *and RIC*[c] (T–C/C) Median (25–75 percentile)	*Ratio* (Tm/Cm) Median (25–75 percentile)
Cat D (pm/mg)	304	1598	0.0000	6.2	7.3
	136 – 309	1122 – 2380		3.7 – 12	4.5 – 13
Cat B_a (mEU/g)	1020	70788	0.0001	60	63
	646 – 2244	35972 – 190910		28 – 179	28 – 184
Cat B (ng/mg)	8	222	0.0000	26	27
	4.2 – 10.3	142 – 342		18 – 77	19 – 78
Cat L_a (mEU/g)	1.9	742	0.0000	157	274
	0.5 – 9.2	103 – 1628		50 – 684	54 – 1012
Cat L (ng/mg)	16	88	0.0000	4.9	5.9
	12 – 22.3	68 – 140		2.6 – 8.2	2.6 – 9.2
Cystatins$_a$ (mIU/g)	468	283	0.0001	– 0.45[d]	0.59
(patients, n=43)	255 – 808	166 – 458		(–0.63) – (–0.21)	0.40 – 0.88
decreased (n=37)	504	258	0.0000	– 0.98	0.54
	309 – 880	138 – 383		(–0.66) – (–0.36)	0.36 – 0.78
increased (n= 6)	177	471	0.028	1.38	2.33
	137 – 317	358 – 5068		0.59 – 1.9	1.59 – 2.88
Stefin A (ng/mg)	108	143	0.019	0.38[e]	1.38
(patients, n= 51)	65 – 194	78 – 390		(–0.3) – 1.4	0.69 – 2.44
decreased (n=20)	143	71	0.0001	– 0.37	0.63
	94 – 221	31 – 103		(–0.74) – (–0.20)	0.26 0.79
increased (n= 31)	76	234	0.0000	3.1	2.19
	54 –172	125–455		0.43– 3.81	1.41 – 4.82

(a) The significance of the differences in median values of tumor and control tissue was calculated by Wilcoxon matched pairs signed-rank test;
(b) RISA: relative increase in specific activity;
(c) RIC: relative increase in protein concentration;
(d) Tm/Cm, ratio between median values in tumors and median values control tissues.

activity and Cat B protein concentration as normalised specific activity and this was much higher in tumors (median value 9.4 nEU/g) than in the control tissues (median value 3.8 nEU/g).

The difference in normalised specific activities in tumors compared to control tissues was even larger for Cat L, 8.4 nEU/g *vs* 0.12 nEU/g, respectively. The data shows that (1) cathepsin activities are upregulated in tumor tissues and are greater than that can be accounted for by the up-regulated protein levels and (2) the increase in Cat L activity in tumors is much higher than that of Cat B. Correlation between the relative increases in cathepsins was also calculated by nonparametric statistical tests, as shown in *Table 3*. Correlation between the increase in activity and the amount of the respective protein was observed for Cat B, but not for Cat L. However, significant correlation (r = 0.60) was established between the increase in Cat B and Cat L activities, while poor correlation (r = 0.43) was established for the respective protein levels. The increase in Cat D protein did not correlate with the increase in concentration of cysteine proteinases. Similar results were observed when only tumor values were compared (not shown).

Cystatins

Table 2. shows that inhibitory activity of cystatins was significantly decreased by a factor of 2 in 37 (out of 43 measured) tumors and was elevated 2.3 fold in 6 cases, still resulting in 1.7 fold significant ($p<0.0005$) decrease in inhibitory activity in total population of tumor samples compared to their control counterparts. It is noteworthy, that cystatin activity in control samples of the group of tumors where an increase was observed was markedly lower than in the control group with decreased inhibitors. Therefore, the relative increase in CPI activity in tumors results also from the down-regulation of cystatins in adjacent stromal tissues. Similarchange was observed for

Table 3. **Correlation between relative increase (ratio T/C) of cathepsins and cysteine proteinase inhibitors in tumors**

*Correlation coefficients (Significance, p)**						
Tumor/Control:	*[Cat D]*	*Cat B_a*	*[Cat B]*	*Cat L_a*	*[Cat L]*	*CPI_a*
[Cat D]	1					
Cat B_a	0.25 (0.90)	1				
[Cat B]	0.19 (0.21)	**0.33 (0.04)**	1			
Cat L_a	0.11 (0.41)	**0.60 (0.000)**	0.09 (0.58)	1		
[Cat L]	0.30 (0.61)	**0.36 (0.028)**	**0.43 (0.009)**	0.17 (0.92)	1	
CPI_a	0.01 (0.93)	0.07 (0.66)	–0.10 (0.58)	–0.04 (0.77)	0.22 (0.22)	1
[St A]	–0.07 (0.62)	–0.06 (0.65)	–0.03 (0.85)	0.06 (0.62)	0.21 (0.21)	0.24 (0.21)

*Correlations were determined by two-tailed Spearman rank test.

stefin A protein, which decreased 1.7 fold in 20 (out of 51 measured) tumors and increased 2.2 fold in 31 tumors compared to controls. Decrease in cystatin activity and in stefin A level in control counterparts was significant and added markedly to the overall increase in T/C ratio. This data suggests that with respect to the levels of endogenous inhibitors, two subpopulations of tumors exist, one where the CPIs are up-regulated and the other, where the CPIs are down-regulated in addition to the opposite regulation in the adjacent stromal tissues. The population with positive T/C ratio in cystatin activity was small in contrast to the population with highly elevated stefin A T/C ratio. Cystatin activity and stefin A did not correlate, although the two groups, with down-regulated and up-regulated stefin A, respectively, differed significantly in the relative increase (T/C) of cystatin activity, as shown in *Fig.1.*

Table 4. **Correlation of biochemical parameters with histopathological and clinical parameters**

Tumor /Control Median (25–75 %)	*[Cat D]*	*Cat B_a*	*[Cat B]*	*Cat L_a*	*[Cat L]*	*CPI_a*	*Stefin A*
Lymph nodes							
negative	6.5	59	21	191	5.8	0.6	1.1
(n = 30)	4.7 – 9.0	19 – 160	14 – 48	26 – 670	4.2 – 8.3	0.4 – 0.9	0.6 – 2.2
positive	8.4	91	42	321	6.3	0.6	1.4
(n = 27)	3.8 – 17	25 – 232	24 – 106	25 – 1261	25 – 10.3	0.4 – 0.8	0.7 – 2.9
Significance (p)	*0.25*	*0.24*	***0.0093***	*0.13*	*0.81*	*0.81*	*0.46*
Stage							
I and IIA	7.2	41	21	225	5.8	0.6	1.1
(n = 25)	4.8 – 10.1	20 – 160	14 – 40	27 – 763	3.8 – 9.0	0.4 – 0.9	0.7 – 2.4
IIB and III	8.3	91	48	321	6.1	0.7	1.4
(n = 32)	3.6 – 16.6	31 – 232	24 – 100	80 – 1097	3.5 – 10.1	0.4 – 0.9	0.8 – 2.9
Significance (p)	*0.69*	*0.08*	***0.0027***	*0.13*	*0.83*	*0.73*	*0.7*
Histological grade							
grade 1 & 2	7.3	33	23	88	5.7	0.6	1.1
(n=24)	4.8 – 10.4	15 – 126	18 – 66	28 – 352	3.5 – 8.6	0–4 – 0.8	0.7 – 2.5
grade 3	7.6	124	39	653	6.3	0.71	1.4
(n = 26)	3.8 – 17.4	40 – 427	24 – 106	109 – 2172	3.6 – 11 3	0.4 – 0.9	0.7 – 3.0
Significance (p)	*0.57*	***0.0046***	*0.06*	*0.055*	*0.57*	*0.31*	*0.61*

* The samples were dichotomised as indicated and median values (25 to 75 percentiles) of the biochemical parameters were calculated. The significance of the differences in the two groups were calculated by Kruskal-Wallis one way ANOVA.

The ratio between median Cat B activity and median cystatin activity was 0.064 in control and 7.35 in tumor tissues, indicating over 115 fold increase in the Cat B:cystatins activity ratio. Similarly, the Cat L:cystatin activity ratio was increased 655-fold in tumors, from 0.004 in controls to 2.6 in tumors.

In the total population of tumors cathepsins did not correlate with the endogenous inhibitors, either with the cystatins inhibitory activity or with stefin A. However, in the stefin A down-regulated group of 20 tumors, we found a significant inverse correlation with Cat B ($r = -0.64$, at $p < 0.006$), while in 31 tumors with up-regulated stefin A significant positive correlation ($r = 0.59$, $p < 0.003$) with Cat B was found.

Correlation with clinical and histo-pathological parameters

The relationship of cathepsins and cystatins to established prognostic factors was evaluated as indicated in *Table 4*. Due to the small number of samples, we grouped tumors only in two subpopulations: Lymph node positive tumors expressed higher values of cathepsins and stefin A, but only Cat B concentration was increased significantly ($p < 0.009$). Very similar trend was observed in high stage tumors, where only the increase in Cat B protein was significant ($p<0.003$).

In contrast to Cat B protein, Cat B activity seemed to be more related to histological grade, as in poorly differentiated tumors Cat B activity was increased about 4 fold ($p<0.005$), while Cat B protein was increased only about 1.5-fold ($p<0.06$). There was no correlation of patient age, tumor size, ER, PR status and the biochemical parameters measured in this study.

Prognostic impact of cathepsins and cysteine proteinase inhibitors

Prognostic significance of the biochemical parameters for disease-free and overall survival was determined by both Cox-univariate analysis and Kaplan-Meier survival curves. The latter is illustrated in *Figure 2 (a, b and c)* showing the disease-free survival curves for the increase in Cat B activity and Cat B protein and a decrease in stefin A, respectively. As shown in *Table 5*, a 3-fold decrease in tumor stefin A was the best prognostic factor, followed by 22-fold increase in Cat B protein and 50-fold increase in Cat B activity. For overall survival rate using the same cut-off points, stefin A was again the most significant prognosticator, followed by Cat B activity and protein concentration. Cat L protein and activity were of borderline significance, while Cat D increase was not significant. Cat B and stefin A were better prognostic indicators than the established histopathological prognosticators.

Table 5. **Prognostic significance for disease free and overall survival: (Kaplan–Meier univariate analysis)**

*Tumor/Control Cut-off**	*Disease-free survival Significance, p*		*Overall survival Significance, p*	
[Cat D]				
not found	n.s.		n.s.	
Cat B_a				
50 fold	0.04	(19/26)*	0.008	(19/28)*
[Cat B]				
22 fold	0.03	(11/29)	0.04	(11/29)
Cat L_a				
50 fold	0.06	(15/35)	0.08	(17/31)
500 fold	0.17	(32/14)	0.06	(32/14)
[Cat L]				
3 fold	0.08	(6/36)	n.s.	
$Cystatins_a$				
0.5 fold (2 fold decrease)	0.12	(14/25)	n.s.	
[Stefin A]				
0.3 fold (3 fold decrease)	0.008	(5/40)	0.02	(5/40)
Tumor size				
(>2.5)	n.s.		0.05	
Lymph nodes				
(+/−)	0.06		0.05	
Stage				
(> IIB)	0.05		0.03	
Histological grade				
(>2)	0.06		0.05	
ER				
(+/−)	n.s.		n.s.	

*patients number in each group below and above the cut-off value. Cut-off values for survival analysis were optimised as shown in *Figure 2*.

Discussion

In this study of sixty matched pairs of tumor and control tissues, we confirmed our previous results on cysteine cathepsins and stefins in breast tumors,[26] where significantly higher induction of Cat L and Cat B activity was observed, compared to the increase in Cat D activity. Here, we have shown that, in addition to enzyme activities, protein synthesis was induced in tumors: Cat B concentration was increased 27-fold, Cat L about 5-fold and Cat D about 6-fold compared to control counterparts. Comparing malignant with control tissue for the same breast, a 10-fold increase in Cat B concentration was found by Gabrijelčič et al[15] in 20 tumor samples. Thomssen et al[41] reported 11-fold increase in Cat B and 7.6-fold increase in Cat L in 167 carcinomas compared to benign breast tissues. Although cathepsins were in a similar concentration range, the mean

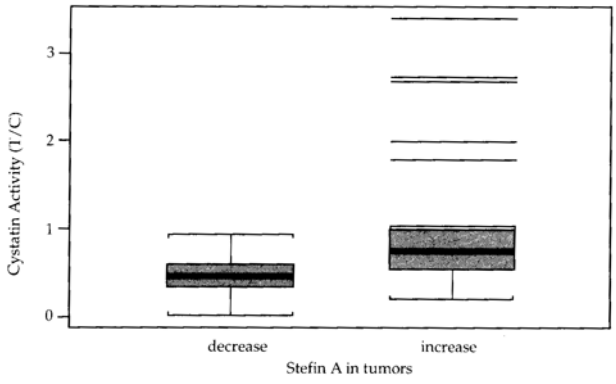

Figure 1. *Correlation between the inhibitory activity of cystatins and stefin A in matched pairs of 51 tumors. Stefin A was decreased in 20 tumors and increased in 31 tumors, compared to the control adjacent non-cancerous tissues. The two groups of tumors, i.e. with decreased and increased levels stefin A, differed in the relative increase in cystatins activities. The difference was highly significant ($p<0.009$) as calculated by Kruskal-Wallis one way ANOVA.*

and median values measured in all these studies differ. This can be explained mostly by differences in the immunospecificity of antibodies used for ELISA kits.

The important new finding in this study is that Cat L and Cat B activities were much more increased than were the respective proteins. When we normalised specific enzyme activity to the enzyme concentration, we observed that a much larger fraction of the enzyme, 9.4 nEU/gCat B, was active in tumors compared to 3.4 nEU/gCat B in controls. An even greater increase was observed for normalised Cat L activity in tumors. This suggests that the increase in Cat L and Cat B activities was not entirely due to the induction of the respective proteins. Enhanced activation of the induced enzymes must have occurred in tumor tissues. Autoactivation and/or activation by another proteinase, for example Cat D, may be enhanced in tumors due to higher levels of Cat D. It was recently demonstrated that the activation of Cat B secreted by a colorectal cancer cell lines required low pH and was mediated by Cat D.[43] It was proposed that an *in vivo* mechanism may exist that allows for Cat D dependent activation of latent procathepsin B outside the cells. Alternatively, less effective inhibition by the endogenous inhibitors in tumors may be responsible for the activation of cysteine cathepsins. Our results also suggest that the activation is more effective for Cat L than for Cat B, possibly due to a more effective binding of Cat L to cystatins, which were mostly down-regulated in tumors. Good correlation (r=0.6) between the relative increase in Cat B and Cat L activities was observed, suggesting a related mechanism of activation. In contrast, poor correlation (r=0.4) was found for the increase in protein expression as also observed previously.[24,41] We suggest that the activation of cysteine proteinases in tumors is enhanced over and above protein levels and that the two cysteine cathepsins are to some extent regulated in a co-ordinated fashion, in contrast to Cat D, where no correlation was observed with cysteine proteinases.[23,24] *In vitro* experiments in transformed human breast epithelial cells[25] also suggested specific regulation and function of each of the three most abundant intracellular cathepsins.

The increase in Cat D protein observed in this study and in Cat D activity reported previously[26] is about five to six fold, indicating that there was no additional activation in tumor tissues. In support of the above proposal is the fact that Cat D protein and activity correlated,[23] but only poor correlation was observed between the concentration and the activities of cysteine cathepsins in breast[24] *(Table 3)* and lung carcinoma.[11,44]

The correlation of cysteine cathepsins with established prognostic factors was only observed for Cat B protein, which was significantly more increased in tumors which had already invaded axillary lymph nodes. As a result of this association, Cat B protein was also significantly more elevated in high stage tumors. When only tumor Cat B protein was related to tumor stage, we found a much less significant (0.06) association, similar to previous studies.[26,41] Our results are not in agreement with the two contradictory reports which first suggested that Cat B correlated with the degree of malignancy[15] and later on significant decrease of Cat B in tumors with invaded lymph nodes.[4] In poorly differentiated tumors, an approximately four fold increase in Cat B activity and nonsignificant increase in Cat B protein was observed, indicating that the activation of Cat B may be linked to cell differentiation. Taken together, the data suggest that the protein induction and Cat B activation are related to different pathophysiological processes during tumor progression. Cat D did not correlate with any of the established prognostic factors, as reported in many studies.[30,33,45]

In contrast to studies reporting the similar levels of cystatins in malignant tissue as in normal tissues[5,12,35] we found significant, about two-fold lower cystatin activity in the total population of breast tumors. Previously, we reported a mean 1.27-fold decrease in cystatin activity although, similar to this study, two groups of patients with downregulated and upregulated cystatin activities, were distinguished, the former expressing significantly higher levels of Cat B and Cat L activities and comprising tumors of high risk patients.[26] The relative increase in cystatin activity was due both to an increase of cystatins in tumors and to their decrease in control tissues indicating a down-regulation of inhibitors in stromal tissues adjacent to tumor, possibly induced by tumor cells. Alterations in stefin A levels were also found in lung parenchyma, adjacent to squamous cell lung carcinoma[12] (and unpublished data). These resulted in more than a hundred-fold increase in ratio between Cat B:

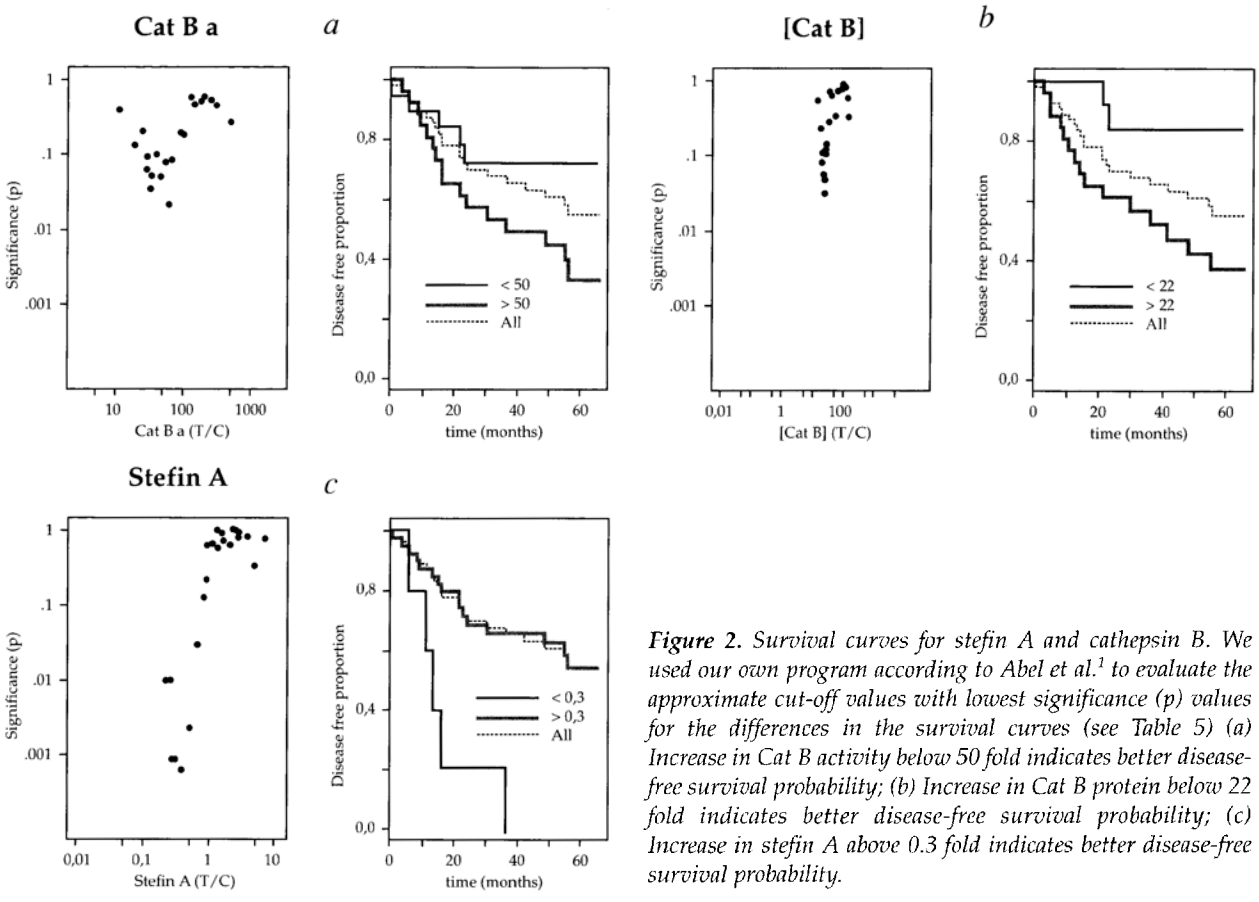

Figure 2. Survival curves for stefin A and cathepsin B. We used our own program according to Abel et al.[1] to evaluate the approximate cut-off values with lowest significance (p) values for the differences in the survival curves (see Table 5) (a) Increase in Cat B activity below 50 fold indicates better disease-free survival probability; (b) Increase in Cat B protein below 22 fold indicates better disease-free survival probability; (c) Increase in stefin A above 0.3 fold indicates better disease-free survival probability.

cystatin and Cat L:cystatin activity in tumors. Increased Cat B and Cat L *vs* cystatins activity was also found in lung tumors and prognostic significance.[11] As in our study, cystatin activity was not related to the established prognostic factors in lung carcinoma, but was, in contrast to our finding, increased in most of the tumors compared to the adjacent lung parenchyma.[11,12] Cystatin activity comprises the activity of various inhibitors, such as stefins A and B, cystatin C and kininogens and recently, the cystatin M gene was discovered and was found to be downregulated in metastatic mammary epithelial tumor cells.[37] Cat D was suggested to inactivate endogenous cystatins and possibly contribute to the observed activation of cysteine cathepsins in tumors.[28] However, we found no correlation between cystatins and Cat D levels.[24]

We reported previously that the average values of stefin A protein and mRNA content were lowered in some, but not all tumor samples, in agreement with the present study, where a significant imbalance in stefin A content in tumor *vs* the adjacent stromal tissue has been observed. Lowered stefin A had an impact on total cystatins activity *(Fig.1)* and was associated with Cat B activity. A one to two fold increase in stefins A and B was reported recently for lung carcinoma, where the increase in total cystatin activity and stefin B indicated good prognosis.[12] It was noteworthy, that the authors did not find stefin A of prognostic value in squamous and adenocarcinoma, the former having significantly higher levels of stefin A. Both, stefins A and B were also found to be significant for prognosis in head and neck cancer.[5] Taken together, our data strongly suggests that an imbalance between CP and cystatins in tumor and possibly in the adjacent control tissue plays an important role in breast tumor progression.

This is the first report demonstrating that a decrease in stefin A was related to a significantly worse prognosis for breast carcinoma patients. The increase in Cat B activity was also good prognostic factor for DFS (disease-free survival) and OS (overall survival), followed by Cat B protein concentration. The data are slightly different from our previous reports, where we suggested that Cat L, but not Cat B, activity was prognostic for breast cancer patients, possibly due to different preparation and storage of breast tumor cytosols, which may have affected the relatively unstable Cat L protein.[42] Prognostic data are also only partially in agreement with the study by Thomssen et al.[41] who found better prognostic significance of Cat L compared to Cat B protein and can be explained mostly by the differences in immunochemical assays used in the two studies. Cat L was also sig-

nificant for prognosis in lung carcinoma in the most recent study by Werle et al.[44] Immunohistochemically determined Cat B was also significant prognostic factor in adenocarcinoma of the lung[18,40] and in brain tumors.[31] We suggest that the levels of Cat B activity and protein in primary tumors may have an impact on prognosis of the disease, although it may not prove to be an independent prognostic factor.

No correlation between Cat D and prognosis was demonstrated in our study, what is in contrast to the majority of clinical studies on predictive and prognostic significance of Cat D in breast carcinoma. Discrepancies between the results of various studies are mostly due to differences in the number and subpopulations of patients, the prevalence and types of treatment used, the length of clinical follow-up, the way in which the data were analysed and the methods used to measure the expression of cathepsins.[30,45]

In conclusion, we report here that the progression of breast cancer is associated with cysteine dependent proteolysis, where at least two enzymes, Cat B and Cat L are highly upregulated, both at the activity and at protein levels. This is partly due to the alteration in endogenous cystatin levels, particularly of stefin A, both in tumor and adjacent stromal tissues. Further clinical studies are needed to evaluate the clinical application of cathepsins and stefins and to understand the mechanisms of regulation of cysteine dependent proteolysis in tumor progression.

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