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Specific oncological contribution of cathepsin D and pS2 in human breast cancer: their relationship with TNM status, estradiol receptors, epidermal growth factor receptor and *neu* amplification

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Abstract

The present study attempts to clarify the specific contribution of cathepsin D (CD) and pS2 to the progression of breast cancer (BC) by examining the relationship between these two factors and TNM status, tumour grade, estradiol receptors (ER) and the prognosis factors epidermal growth factor receptor (EGFR) and *neu* amplification in a group of 270 BC patients. CD and pS2 were determined by an immunoradiometric procedure in tumour cytosols obtained for ER. *Neu* amplifications were evaluated by dot-blot, in tumour DNA. EGFR was determined in membrane tumour preparations obtained from ER cytosols by a two-point radiometric saturation assay. CD is basically related to bad prognosis factors and has a direct correlation with tumour size ($P = 0.025$) and EGFR content ($P = 0.007$) and is associated with the presence of metastases ($P = 0.000$). pS2 is mostly related to good prognosis factors and showed an inverse correlation with the Scarff-Bloom Index ($P = 0.011$) and a

Abbreviations: ANOVA, analysis of variance; BC, breast cancer; BRI, Bloom-Richardson Index; CD, cathepsin D; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estradiol receptors; MAV, multifactor analysis of variance; MLRA, multiple linear regression analysis; RFS, recurrence-free survival; TP, total proteins.

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direct correlation with ER content (P = 0.014). Finally, pS2 and CD also showed a strong mutual association ($P = 0.009$) and the fact that both correlated with ER content confirms in tumours the experimental finding that they are estrogen-induced proteins.

Keywords: Cathepsin D; Breast cancer; pS2; TNM status; Estradiol receptors; Epidermal growth factor receptor; *Neu* amplification

1. Introduction

Cathepsin D (CD) is an acidic protease present in all cells, which is secreted as a 52 kDa precursor and the production of which in breast cancer (BC) cells is stimulated by estrogens [1]. High CD concentrations have been found in cancer cells and this overexpression is believed to allow the tumour cells to digest the extracellular matrix, thus facilitating tumour metastasis [1]. This suggests that high CD levels might indicate greater risk for tumour spread.

pS2 is a 6.4 kDa polypeptide secreted by the MCF-7 breast cancer (BC) cells and many human BC [2]. The biological significance of this polypeptide remains unknown. The production of pS2 is estrogen-inducible, since the addition of estrogen to cultures of MCF-7 increases the secretion of pS2 [3]. A great many studies on BC coincide in pointing out that pS2-positive BCs are usually ER-positive [4–10]. Therefore, pS2 implies not only the presence of ER, but is also an exponent of its biological activity, and the presence of pS2 makes it possible to classify the BC group of positive estradiol receptors (ER+) as being the ER+/pS2+ phenotype, those in which a better response to anti-estrogenic treatment is expected [6].

There are univariate studies which related CD or pS2 with tumour parameters, grade [4,11], presence of steroid receptors [4–11] and with pS2 or CD [4,5,11]. Although multivariate analysis between CD or pS2 and the common prognosis factors has been reported, there is, however, a need for multivariate analysis in which the relationship between CD and pS2 and the conventional prognosis factors (T, N, M, histological grade, age, menopausal status, ER or PR status) and second generation prognosis factors, such epidermal growth factor receptor (EGFR) or *c-erbB-2*/HER-2/*neu* amplification, are considered together. The scarcity of such studies makes it difficult to ascertain what specific contribution is made by CD or pS2 to the later generation tumour prognosis factors, such as EGFR and/or *neu* amplification. Hence, in an attempt to clarify the specific contribution of CD or pS2 to BC progression, the present study examines the relationship between these two factors and TNM status, tumour grade, ER, EGFR and *c-erbB-2*/HER-2/*neu* amplification in a group of 270 BCs. Preliminary evaluation of the prognosis significance of CD and pS2 is also considered.

2. Material and methods

2.1. Patients

The study included 270 BCs from consecutive patients. Sixty-nine carcinomas were from premenopausal women, with a median age of 41 years and a range of 24–49 years and 201 from postmenopausal women, with median age of 64 and a range of 42–89. The median follow-up period was 13 months with a range of 3–32 months.

Tumour samples were obtained at the time of mastectomy or tumorectomy and were stored in liquid N_2 until analysed. In each sample CD, pS2, ER, EGFR and *neu* amplification were determined.

2.2. Methods

Cathepsin D and pS2 kits (ELSA-CATH-D, ELSA-PS2; CIS Bio International). Tritium labelled estradiol, 2,4,6,7-^{3}H 17β-estradiol (E_2-^{3}H), 91 Ci/mmol (Amersham). Iodine labelled epidermal growth factor ([^{125}I]EGF), 164–178 μCi/μg (New England Nuclear). Cold EGF (Biomedical Technologies Ltd). DNA labelling and detection non-radioactive kit (Boehringer Mannheim). Hybond N nylon membranes (Amersham), Proteninase K (Boehringer Mannheim), AMPPD (3-2-spiroadamantane)-4-methoxy-4 (3-phosphoryloxy) phenyl-1,2-dioxiethane (Boehringer Mannheim).

2.3. Histopathology and TNM status

The tissue sections were stained with haematoxylin-eosin and, in 208 tumours, the degree of differentiation was assessed in accordance with the Bloom-Richardson Index [12] (BRI) and the tumours were grouped into three grades: grade I comprised scores 3–5, grade II scores 6–7 and grade III scores 8–9.

Two hundred and twenty-nine cancers (85%) were classified as ductal carcinoma, 23 (9%) as lobular carcinoma, 12 (4%) as medullary carcinoma and the 6 remaining BC were distributed among papillary, tubular and colloid carcinomas. Almost half of the tumours (126/266) were T2, while the rest were distributed among the other sizes (see Table 1); 153 patients had nodal involvement (N+) at the time of the mastectomy and 15 patients had distant metastases.

2.4. Cathepsin D and pS2 assays

Both CD and pS2 were determined in the tumour cytosols obtained for ER assays, which were stored at −20°C until analysis. The cytosols had to have

between 1 and 2 mg/ml total proteins (TP) and those with TP below the lov limit were discarded. The method followed for CD, ELSA-CATH D (Cis L. International), has been evaluated by the EORTC and considered adequate for clinical purposes [13]. For pS2, a kit of the same brand, ELSA-pS2 (CIS International), was used. In every assay the evaluated control sample provided in the kit was included. Final results were expressed in ng of CD or pS2 per mg TP [14]. The between-assay precision, obtained by including the same sample in different assays, gave C.V. values of 6.2% and 7.6% for pS2 and cathepsin D, respectively.

2.5. Positivity criteria

According to the results on the CD concentration, the BC were classified as negative (CD–), borderline (CD±) or positive (CD+) as follows: CD lower than 30, between 30 and 60, or higher than 60 ng/mg TP, respectively. The BC were classified as pS2 negative (pS2–) or positive (pS2+) depending on whether the pS2 concentration was lower than 30 ng/mg TP (pS2–), or was equal to or higher than this limit (pS2+).

2.6. Neu amplification

Neu amplification was evaluated from BC DNA. The procedure followed has been described previously [15] and, briefly, consists of a digestion of homogenized tumour samples with proteinase K. DNA was purified by the phenol-chloroform procedure with a final precipitation step with cold ethanol [16]. The DNA obtained was loaded on a Hybond membrane with the help of a dot manifold (Biorad). Each sample was tested in triplicate with increasing dilutions (1:1, 1:4 and 1:8). The hybridization was done with a digoxigenin-labelled probe [17] of the *Hin*d III digested pCD plasmid containing an insert of the human *neu* sequences (ATCC 57584). The amplification was evaluated by comparison of the intensity of each dilution of each sample with a reference placental DNA, which was taken as the reference of negativity, and a sample of DNA + pCD plasmid, which was a reference for *neu* amplification [15]. A tumour was considered to have a *neu* amplification (*neu*+) when the intensity of any of the more diluted loadings was equal to or greater than the lower dilution of placental DNA.

2.7. EGFR quantification

EGFR was determined in the membrane preparation obtained from the cytosol [18]. The EGFR assay [18] involves incubating duplicate samples of 100 μl of plasma membranes plus 100 μl [^{125}I]EGF (40 000 counts/min, 200–300 fmol/ml) and 200 μl of assay buffer to evaluate the total binding, or with 200 μl of a solution of EGF in assay buffer containing 8333 fmol of

cold EGF, to evaluate the non-specific binding. The samples were incubated at room temperature for 2 h and, then centrifuged at 20 000 × *g* for 10 min at 4°C. The specific binding was obtained by subtracting the mean counts/min of non-specific binding from that of total binding. Results were expressed in terms of fmol [^{125}I]EGF bound to plasma membranes/mg plasma membrane TP. Homogenates of placental tissue (rich in EGFR) were used in each assay as the positive control.

An EGFR concentration greater than 0.5 fmol/ml homogenate, with the non-specific binding lower than 70%, was used as a criterion of positivity (EGFR+) [18]. Final results were given in fmol EGFR/mg TP of membrane.

2.8. ER quantification

ER was quantified by Leake's method [19]; 50 μl of [^{3}H]E_2, with or without 100 times diethylstilbestrol, in seven increasing concentrations in the 10^{-10}–10^{-9} mol/l range were added to 150 μl of cytosolic fraction. All the tubes were incubated at 4°C for 18 h. The [^{3}H]E_2-receptor complex was separated from the free [^{3}H]E_2 by the dextran-coated charcoal method. The results were expressed in fmol ER/mg TP.

The results obtained with the ER method were validated by the quality control program established by the EORTC Receptor Study Group Quality Control and by verifying that our results lay within the mean ± 1.5 S.D. of the results of the participants using equivalent methods.

2.9. Statistics

The proportions of positives were compared by χ^2. Kruskall-Wallis non-parametric univariate analysis of variance (ANOVA) [20] was used to compare the results of two or more groups. Multifactorial analysis of variance (MAV) was used to evaluate the specific effect of the parameters studied. Multiple linear regression analysis (MLRA) was used to correlate a quantitative dependent variable with several qualitative independent variables. Most of the statistical procedures were carried out using the statistical package program Statgraphics. Kaplan-Meier univariate analysis for recurrence-free survival (RFS) with log-rank test for the CD and pS2 results, stratified according to the patient's notal status, used the statistical package SSPS.

3. Results

3.1. Cathepsin D

3.1.1. Univariate analysis

There is a significantly larger proportion of CD+ or CD± BC among the larger size tumours ($P = 0.07$; Table 1). These BCs also showed significantly

higher concentrations of CD than those with smaller tumour sizes ($P = 0.01$; Table 1 and Fig. 1A).

The CD concentration was also significantly higher in BC with metastases (M1) at the moment of tumour excision, than in those without metastases (M0) ($P = 0.018$; Table 1 and Fig. 1B). This was not caused by the existence of a larger proportion of CD+ BC among the M1 BC, but was due to the extremely high CD concentrations, 169 and 195 ng CD/mg TP, found in the two BC CD+.

There was a significantly larger proportion of CD+ or CD± BC among SBI II or III BC than among SBI I BC ($P = 0.04$; Table 1), but, no statistical differences in the CD content of the BCs, grouped according to their SBI, was observed (Table 1).

The proportion of CD+ or CD± BCs was significantly larger among EGFR+ BCs than in EGFR− BCs ($P = 0.009$; Table 1). This explains the parallel trend observed in the tumour CD concentration ($P = 0.02$; Table 1).

The presence of CD+ or CD± BC was significantly greater in the group of *neu*+ BCs than in the *neu*− BCs ($P = 0.023$; Table 1). This explains why the CD concentration was also significantly increased in the group of *neu*+ BCs in comparison with the *neu*− BCs ($P = 0.02$; Table 1).

3.1.2. Multivariate analysis

In the MAV, in which the concentration of CD was analysed in relation to the qualitative parameters (menstrual status, TNM status, SBI and pS2, EGFR and ER status), only pS2 and M remained as significant variables to explain BCs CD content. However, the associations with T and EGFR status observed in ANOVA, were lost (Table 2).

A MLRA of the log(CD+1), taken as a dependent variable, in relationship with the independent parameters (age, tumour size in cm, percentage of nodes involved (not grouped SBI) and log(ER+1), log(pS2+1) and log(EGFR+1)) was found (Table 3). In this analysis only tumour size, ER and EGFR were significantly correlated with CD (Table 3).

3.1.3. Prognostic relevance of cathepsin D

The actuarial Kaplan-Meier analysis for RFS obtained by grouping the BCs dichotomically in CD+ and CD−, including the CD− and CD± BCs in the CD− group and stratifying by N did not show statistical significance (Log Rank = 0.28; $P = 0.59$).

3.2. pS2

3.2.1. Univariate analysis

The pS2 levels of premenopausal BC patients were significantly higher than those of postmenopausal women ($P = 0.03$, Table 4).

Table 1
Breast cancer cathepsin D (CD) status or concentrations and pathological parameters or biological tumour marker status

Parameter	CD− N	CD± N	CD+ N	χ^2;	*P*	Mean ± S.D. (*N*) mg CD/mg TP	Median (range) mg CD/mg TP	KW;	*P*
Premeno	38	25	6	0.56;	0.75.2	34 ± 26 (67)	27 (141)	0.42;	0.51
Postmeno	105	72	24			36 ± 26 (197)	29 (193)		
T1	48	16	7	11.68;	0.07	28 ± 21 (70)	23 (92)	11.26;	0.01
T2	62	52	12			36 ± 23 (121)	32 (136)		
T3	5	7	3			42 ± 21 (15)	43 (67)		
T4	26	20	8			41 ± 38 (53)	34 (195)		
N−	63	37	10	2.04;	0.36	32 ± 23 (108)	27 (139)	1.94;	0.16
N+	75	58	58			38 ± 29 (148)	33 (195)		
M0	104	73	19	0.96;	0.61	34 ± 23 (191)	29 (140)	5.57;	0.018
M1	6	7	2			63 ± 54 (15)	45 (181)		
SBI I	47	18	12	10.14;	0.04	37 ± 25 (76)	25 (166)	0.11;	0.94
SBI II	45	35	9			33 ± 21 (86)	31 (88)		
SBI III	22	19	1			32 ± 19 (41)	28 (97)		
pS2−	126	89	22	7.85;	0.02	34 ± 24 (231)	29 (195)	2.32;	0.12
pS2+	15	8	8			46 ± 37 (30)	34 (159)		
ER−	63	41	13	0.13;	0.93	34 ± 25 (113)	28 (122)	0.37;	0.540
ER+	79	56	18			36 ± 28 (150)	30 (194)		
EGFR−	121	67	25	9.35;	0.009	34 ± 26 (207)	27 (169)	5.11;	0.02
EGFR+	20	29	5			40 ± 28 (53)	38 (191)		
Neu−	71	50	15	7.47;	0.023	35 ± 24 (134)	29 (139)	5.90;	0.02
Neu+	7	8	7			53 ± 42 (21)	43 (188)		

N, number of cases; S.D., standard deviation; KW, the non-parametric ANOVA Kruskall-Wallis test; SBI, Scarff-Bloom Index; TP, total protein. Only detectable values were included for computing mean and median statistical parameters.

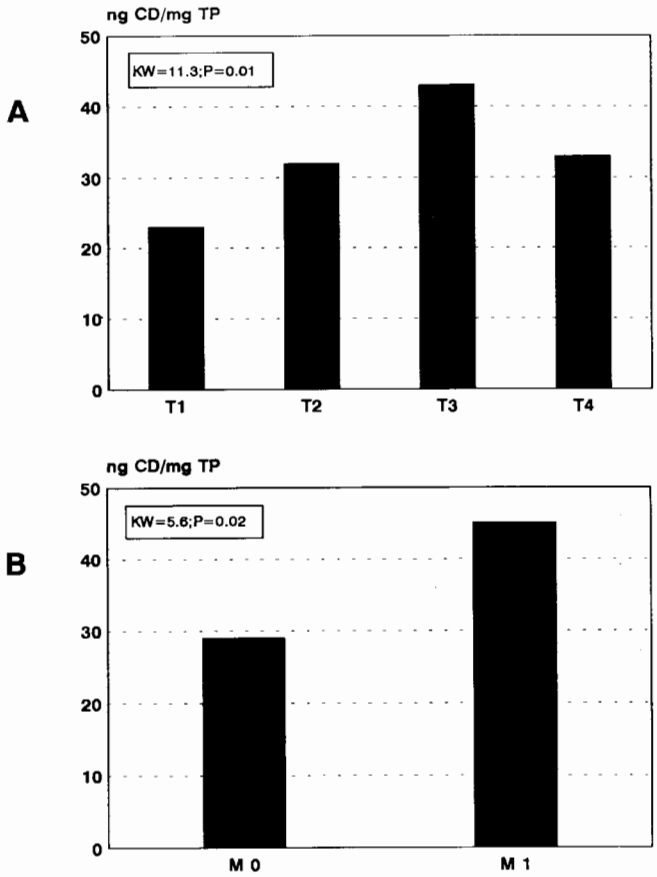

Fig. 1. Relationship of the median cathepsin D (CD) BC content with T (A) or with M (B). KW indicates the non-parametric ANOVA Kruskall-Wallis test (the figures represented here are those shown in Table 1).

The proportion of pS2+ BCs decreases with the SBI ($P = 0.003$, Table 4). This explains the inverse relationship between the pS2 concentration and SBI ($P = 0.002$, Table 4 and Fig. 2A).

The proportion of pS2+ BCs was significantly larger among CD+ or CD± BCs than in the CD− BCs ($P = 0.002$; Table 4), but the CD+ or CD± BCs did not show higher pS2 concentrations than the CD− BCs (Table 4).

All but five of the pS2+ BC (26/31) were ER+ ($P = 0.002$, Table 4). This result is in accordance with the significantly higher pS2 concentration found in ER+ BCs than in ER− BCs ($P = 0.000$, Table 4 and Fig. 2B).

There is a significantly higher pS2 concentration among the EGFR− BCs ($P = 0.02$, Table 4), due to the fact that the large majority of pS2+ BCs (25/30) were EGFR−.

Table 2
Multifactorial analysis of variance of cathepsin D (CD)/pS2 with different tumour parameters

Parameters	Cathepsin D		PS2	
	F	*P*	*F*	*P*
Main effects	3.51	0.0003	2.98	0.001
Menstrual status	0.49	0.49	0.08	0.77
T	1.77	0.15	1.20	0.31
N	1.29	0.25	0.49	0.49
M	17.65	0.00	0.00	0.99
SBI	0.03	0.96	2.62	0.07
ER status	1.04	0.31	3.97	0.05
pS2/CD status	11.58	0.0009	7.42	0.0009
EGFR status	0.30	0.58	0.49	0.49
Global	2.82	0.008	3.51	0.003

3.2.2. Multivariate analysis

When the pS2 concentration was analyzed in relationship with menstrual status, TNM status, SBI, EGFR, CD and ER status, only CD and ER were statistically significant and a trend for SBI was observed (Table 2). However, the significant relationships with menstrual status and EGFR, observed on the ANOVA, were lost here (Table 2).

Table 3
Multiple linear correlation of cathepsin D (CD)/pS2 with prognosis factors

Parameters	Cathepsin D		PS2	
	t	*P*	*t*	*P*
Age	−0.31	0.760	−2.65	0.009
Tumour size (cm)	2.26	0.025	−0.25	0.799
% nodes involved	0.17	0.862	1.97	0.051
SBI	−0.61	0.544	−2.56	0.011
Log(ER+1)	2.25	0.025	2.48	0.014
Log(CD/pS2+1)	1.37	0.169	1.37	0.169
Log(EGFR+1)	2.71	0.007	−0.57	0.564
Global	$R = 0.12$; $N = 165$	$F = 2.82$; $P = 0.008$	$R = 0.15$; $N = 155$	$F = 3.83$; $P = 0.001$

Table 4
Breast cancer pS2 concentrations or status and pathological or biological parameters

Parameter	pS2– N	pS2+ N	χ^2; P	Mean ± S.D. (N) MgPS2/mg TP	Median (range) MgPS2/mg TP	KW; P
Premeno	58	11	1.58; 0.21	16 ± 21 (64)	6 (86)	4.8; 0.03
Postmeno	175	20		14 ± 25 (172)	4 (171)	
T1	58	11	1.76; 0.62	18 ± 30 (62)	5 (170)	1.0; 0.78
T2	110	14		14 ± 21 (107)	6 (164)	
T3	14	1		12 ± 31 (14)	4 (118)	
T4	47	5		12 ± 17 (49)	4 (79)	
N–	95	12	0.04; 0.84	14 ± 25 (91)	4 (171)	0.51; 0.47
N+	132	18		14 ± 23 (138)	5 (164)	
M0	166	24	0.006; 0.93	14 ± 23 (168)	5 (171)	0.19; 0.65
M1	13	2		23 ± 44 (15)	4 (163)	
SBI I	61	16	11.27; 0.003	22 ± 29 (70)	12 (171)	12.51; 0.002
SBI II	78	9		12 ± 19 (78)	4 (120)	
SBI III	41	0		6 ± 6 (35)	3 (25)	
CD–	126	15	7.85; 0.02	14 ± 23 (131)	4 (164)	4.2; 0.12
CD+	89	8		11 ± 15 (82)	4 (85)	
CD±	22	8		27 ± 39 (27)	6 (170)	
ER–	108	5	10.27; 0.002	9 ± 16 (92)	3 (87)	15.6; 0.000
ER+	125	26		18 ± 27 (144)	6 (171)	
EGFR–	183	25	0.27; 0.59	15 ± 25 (193)	5 (171)	9.3; 0.02
EGFR+	48	5		9 ± 17 (41)	2 (86)	
Neu–	113	19	045; 0.50	17 ± 29 (115)	5 (171)	0.006; 0.98
Neu+	20	2		14 ± 21 (20)	5 (85)	

N, number of cases; S.D., standard deviation; KW, the non-parametric ANOVA Kruskall-Wallis test; SBI, the Scarff-Bloom Index; TP, total protein. Only detectable values were included for computing mean and median statistical parameters.

A significant MLRA between the log(pS2+1), taken as a dependent variable, with the independent variables (age, tumour size in cm, percentage of nodes involved, non-grouped SBI and log(ER+1), log(CD+1) and log(EGFR+1)) was found (Table 3). In this MLRA only age, percentage of nodes involved, SBI and ER remained as independent variables (Table 3).

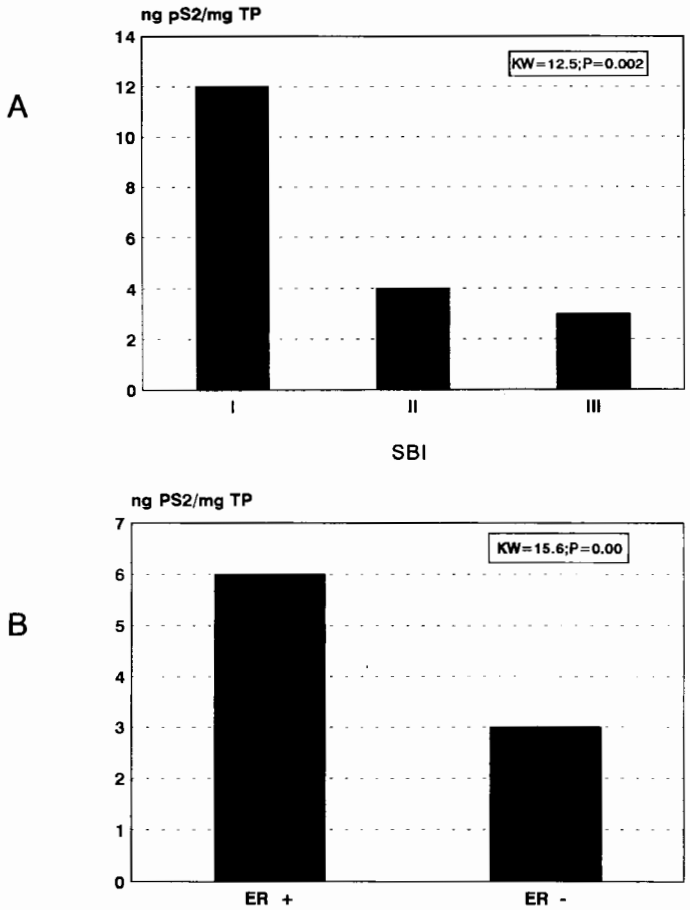

Fig. 2. Relationship between pS2 BC content and the Scarff-Bloom Index (SBI) (A) or with ER status (B). KW indicates the non-parametric ANOVA Kruskall-Wallis test (the figures represented here are those shown in Table 4).

3.2.3. Prognostic relevance of pS2 status

The actuarial Kaplan-Meier analysis obtained for RFS for pS2 stratified by N reached the boundaries of statistical significance (Log Rank = 1.86; $P = 0.17$), which is explained by the trend observed in the N–/pS2+ BCs subgroup (Log Rank = 2.7; $P = 0.1$) to have longer DFS periods in comparison with the pS2–/N– patients.

Discussion

Although most of the conclusion of the present study concern the relationships between CD or pS2 and the most commonly used prognostic variables

of breast cancer, the short follow-up period (13 months), renders the results obtained provisional, and will only be consolidated if they coincide with the results observed after monitoring the patients for longer follow-up periods.

The higher concentration of pS2 found in premenopausal BC cannot be attributed to a greater proportion of pS2+ BCs among the premenopausal women ($\chi^2 = 1.58$; $P = 0.21$; Table 4), but are probably due to their higher levels of circulating estrogens, which induce the ER+ cells to produce higher levels of pS2. This interpretation is also supported by the significant partial correlation coefficient with age found in the MLRA, in spite of the fact that ER was also considered in the correlation (Table 3). This suggests that the relationship between pS2 and age is independent of the tumour ER concentration. A similar trend was shown in a preceding study [21]; however, in other work on the same subject no associations between pS2 and age or menopausal status were found [11].

While in the present study a strong association, both qualitative and quantitative, between CD and T was found, other studies revealed no association [4,5,11]. Although the statistical significance of this association is lost in the MAV, in the MLRA, the tumour size partial correlation coefficient remains significant (Table 3). The finding of the present study coincides with the bad prognosis attributed to the CD. These results suggest that the higher concentration of CD might represent a late event in tumour growth.

The presence of higher CD values among the M1 BC might indicate the potential metastasizing capability attributed to this enzyme and might suggest that the tumours with extremely high CD concentrations could have a greater metastasizing capacity. Moreover, the association between extremely high CD concentrations and metastases seems to be a specific characteristic since metastases remained statistically significant in MAV in the presence of the other parameters analyzed.

Unlike the situation with CD, there was no relationship between pS2 and TNM status. This finding coincides with most of the studies in the field [5,6,10,11].

The inverse relationship between pS2 concentration and SBI had been shown in some preceding studies [4,11,22] and it is consistent with the good prognosis attributed to pS2. On the other hand, this strong association is a specific peculiarity of pS2, for it can be seen in both MAV and MLRA (Tables 2 and 3). In contrast with what was seen for pS2, for CD there was a direct proportional increase in CD+ and/or CD± BCs with SBI, as is also reported in other studies [4,11,23] and this is consistent with the bad prognosis characteristic of CD.

The strong association between CD and pS2 status is a specific trait because it remained in the MAV. On the other hand, this relationship might be explained by the fact that both genes are estrogen-inducible through the ER [3], as has been shown in experimental studies [4,5,11].

The strong association between pS2 and ER+ BCs is a specific characteristic, for it was confirmed by MAV (Table 2) and has been demonstrated in most studies [4–9,11]. However, in spite of this, most pS2+ BCs are ER+, but not all the ER+ BCs are pS2+ [6,7].

Although in the ANOVA no association between CD and ER was found, a direct linear correlation between CD and the log(ER+1) in the MLRA was detected (Table 3). This may indicate that, unlike what is reported for pS2, the synthesis of CD is more dependent on ER tumour content than on the plasma levels of estradiol. The results of the present study are confirmed by some earlier ones [4,5,10], even though in the others an inverse relationship with ER or PR status was found [11,23].

The strong, direct, qualitative and quantitative association between CD and EGFR status, corroborated by MLRA (Table 3), is consistent with the bad prognosis attributed to CD. However, the findings of the present study were not confirmed by a preceding one [10].

Although there was not a large proportion of pS2+ BCs among the EGFR– BC, the majority of the BCs in this group had higher average pS2 concentrations than the EGFR+ BCs. The inverse relationship between pS2 and EGFR found in the present study is consistent with its good prognosis feature.

The higher CD concentration found in BC *neu*+ is explained by the larger proportion of CD+ in this group and supports the characteristic bad prognosis attributed to this protein. Similar findings were reported in a preceding study [23,24], but in yet another [25] no association between the two parameters was found.

The lack of association between pS2 and *neu* amplifications was also found in a preceding study [7] and is consistent with the opposite prognosis significance of the two parameters.

Although the short follow-up period of only 13 months does not allow us to draw solid conclusions, the results of the present study showed no significant prognosis value for CD like that of Ravin et al. [26] and unlike other studies [24,27]. In the case of pS2, a trend toward longer disease-free periods among pS2+ BCs was observed, which coincides with other studies in this field [22] and can be probably attributed to the ER+ character of these BCs.

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References

[1] Rocheford H. Cathepsin D in breast cancer. Breast Cancer Res Treat 1990;16:3–13.
[2] Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of

cDNA sequences of hormone regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res 1982;10:7895–7903.

[3] Brown AMC, Jeltsch JM, Roberts M, Chambon P. Activation of pS2 gene transcription in primary response to estrogen in human breast cancer cell line MCF-7. Proc Natl Acad Sci (Wash) 1984;81:6377–6348.

[4] Gazin JL, Gosselin P, Boniface B, Boniface M, Demaille A. Expression of pS2 and cathepsin D in breast cancer. Int J Oncol 1993;2:1081–1084.

[5] Marsigliane S, Biscozzo L, Correale M et al. Immunoradiometric detection of pS2 and total cathepsin D in primary breast cancer biopsies: their correlation with steroid receptors. Br J Cancer 1994;69:550–554.

[6] Correale M, Paradiso A, Abbate I et al. Cytosolic levels of estrogen-regulated pS2 protein in breast cancer: correlation with tumor proliferative activity. Tumor Biol 1993;14:30–37.

[7] Rio MC, Bellocq JP, Gairard B et al. Specific expression of pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. Proc Natl Acad Sci USA 1987;84:9243–9247.

[8] Foekens JA, Rio MCh, Seguin P et al. Prediction of relapse and survival in breast cancer patients by pS2 protein status. Cancer Res 1990;50:3832–3837.

[9] Koerner FC, Goldberg DE, Edgerton SM, Schwartz LH. pS2 protein and steroid hormone receptors in invasive breast carcinomas. Int J Cancer 1992;52:183–188.

[10] Sacks NPM, Smith K, Norman AP, Greenall M, Le Jeune S, Harris AL. Cathepsin D levels in primary breast cancers: relationship with epidermal growth factor receptor, oestrogen receptor and axillary nodal status. Eur J Cancer 1993;29A:426–428.

[11] Foekens JA, van Putten WLJ, Portengen H et al. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol 1993;11:899–908.

[12] Bloom HJG, Richardson WW. Histologic grading and prognosis in breast cancer. Br J Cancer 1957;11:359–377.

[13] Benraad TJ, Geurts-Moespot A, Sala M, Piffanelli A, Ross A, Foekens JA, EORTC Receptor Study Group. Quality Control of Cathepsin D measurement by the EORTC Receptor Study Group. Eur J Cancer 1992;28:72–75.

[14] Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 1976;72:248–254.

[15] Bolufer P, Molina R, Ruiz A, Hernandez M, Vazquez C, Lluch A. Estradiol receptor in combination with neu oncogene amplification might define new subtypes of breast cancer. Clin Chim Acta 1994;229:107–122.

[16] Maniatis T, Fritsch E, Sambrook J. Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory, 1982.

[17] DNA Labelling and detection nonradioactive. Applications manual. West Germany: Boehringer Mannheim GmbH Biochemica, 1989.

[18] Bolufer P, Miralles F, Rodriguez A et al. Epidermal growth factor receptor in human breast cancer: correlation with cytosolic and nuclear ER receptors and with biological and histological tumor characteristics. Eur J Cancer 1990;26:283–290.

[19] Leake RE, Laing L, Smith DC. A role for nuclear estrogen receptors in prediction of therapy regime for breast cancer patients. In: King RJB, ed. Steroid receptor assays in human breast tumors: methodological and clinical aspects. Cardiff: Alpha Omega Press, 1979;75–86.

[20] Armitage P. Statistical methods in medical research. Oxford, London: Blackwell Scientific Publications, 1977.

[21] Pujol P, Maudelonde T, Daures JP et al. A prospective study of the prognostic value of cathepsin D levels in breast cancer. Cancer 1993;1:2006–2012.

[22] Speiser P, Stolzlechner J, Haider K et al. pS2 protein status fails to be and independent prognostic factor in an average breast cancer population. Anti Res 1994;14:2121–2130.
[23] Tetu B, Brisson J, Côté Ch, Brisson S, Potvin D, Roberge N. Prognostic significance of cathepsin D expression in node-positive breast carcinoma: an immunohistochemical study. Int J Cancer 1993;55:429–435.
[24] Fernö M, Baldetorp B, Borg A et al. Cathepsin D, both prognostic factor and a predictive factor for the effect of adjuvant tamoxifen in breast cancer. Eur J Cancer 1994;30A:2042–2048.
[25] Brouillet JP, Theillet Ch, Maudelonde T et al. 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. Eur J Cancer 1990;26:437–441.
[26] Ravdin PM, Tandon AK, Allred DG et al. Cathepsin D by western blotting and immunohistochemistry: failure to confirm correlations with prognosis in node-negative breast cancer. J Clin Oncol 1994;12:467–474.
[27] Spyratos F, Brouillet JP, Defrenne A et al. Cathepsin D: an independent prognostic factor for metastasis of breast cancer. Lancet 1989;2:1115–1118.