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L. Nakopoulou · A.C. Lazaris · D. Baltas
I. Giannopoulou · N. Kavantzas · A. Tzonou

Prognostic evaluation of oestrogen-regulated protein immunoreactivity in ductal invasive (NOS) breast cancer

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Abstract Determination of steroid receptors and several oestrogen-regulated proteins in mammary carcinomas is useful in the prediction of their evolution and of the likely success of endocrine therapy. Cathepsin D (Cat D), pS2 peptide and heat shock protein 27 (Hsp 27) were detected immunohistochemically in 63 infiltrating ductal (NOS) breast carcinomas, and our results were qualitatively correlated with several clinicopathological indicators and patients' overall survival. Cat D immunostaining of tumour cells was strongly associated with axillary nodal involvement (P_f = 0.0005) and so, it is directly connected with the metastatic capacity of malignant cells. pS2 immunoreactivity was correlated with oestrogen and progesterone receptor positivity (P_f = 0.0009 and P_f = 0.05 respectively) and, nonsignificantly, with good differentiation of the tumours (P_f = 0.06). Neoplastic cells expressing this protein are therefore characterised by a highly organised state of cellular physiology. Hsp 27 was expressed predominantly in tumours with one to four infiltrated lymph nodes (P_t = 0.05), and Hsp 27-positive patients were inclined to rather short survival, possibly due to chemotherapy resistance. In future, prognostic estimation of each one of the examined markers should be performed in specific large subgroups of patients. The findings of this study contribute to the establishment of criteria by which these subgroups should be formed.

Key words Cathepsin D · pS2 · Heat shock protein 27 · Immunohistochemistry · Breast cancer

Introduction

The improvement of methodologies and the progressive increase in our basic knowledge of breast cancer biology has allowed standardisation of the more traditional cellular markers [25], such as oestrogen receptors (ER), ploidy, cell kinetics and ER-associated proteins [8]. A number of more recently considered indices of biological activity have also been examined. Cathepsin D (Cat D) is an oestrogen-induced glycoprotein, the mature active form of which normally functions in the lysosomes at acidic pH and has both growth-promoting and proteolytic activity. The latter associates Cat D expression with increased metastatic potential of tumour cells [9], since passage through basement membranes is thought to depend on the ability of the cancer cell to degrade the proteins of which this barrier is composed via the secretion of proteolytic enzymes [11]. However, this view is criticised [12], and the controversy has not been totally resolved for breast cancer.

The oestrogen-inducible pS2 gene, originally isolated from a breast cancer cell line [18], is correlated with favourable outcome of hormone-dependent female breast tumours. This gene has been cloned and the encoded polypeptide identified as an 84-amino-acid secreted protein whose functions are still ambiguous [24].

Heat shock proteins (Hsp) are inducible proteins expressed in cells only during stressful conditions. Aberrantly functioning cells, such as cancer cells, often constitutively express Hsp which enable them to survive. Hsp 27 is a small Hsp (molecular weight 27 000) initially identified in human breast tumour cells as an oestrogen-responsible protein. Higher amounts of Hsp 27 are expressed mainly in oestrogen target organs of the female reproductive tract [4, 27]. It is a fact that concurrent administration of hyperthermia and chemotherapy increases tumour cell killing. Nevertheless, when malignant cells are exposed sequentially to nonlethal heat shock and then to chemotherapy, the latter is less effective than chemotherapy administered to tumour cells never exposed to nonlethal heat shock. Heat-induced Hsp may thus be involved in drug resistance [19].

In this investigation we examined 63 invasive, not otherwise specified (NOS) mammary carcinomas with regard to the above-mentioned proteins' immuno-expression and looked for any prognostic significance of these

L. Nakopoulou (✉) · A.C. Lazaris · D. Baltas · I. Giannopoulou
N. Kavantzas · A. Tzonou
Department of Pathology, Medical School of Athens University, 75 Mikras Assias Str., GR-115 27 Athens, Greece

markers by correlating our results with various clinicopathological predictors and patient survival.

Materials and method

Patient population

Sixty-three women with operable primary ductal infiltrating NOS breast cancer were evaluated. Being interested in the clinical applicability of all three markers, we tried to work on a histologically homogeneous group of neoplasms:

1. Only stage I-II ductal infiltrating breast carcinomas were studied: tumour no more than 5 cm in greatest dimension, no infiltrated, homolateral axillary nodes fixed to one another or to other structures, no clinical evidence of distant metastasis.
2. No previous or concomitant malignancies of other organs.
3. No patient over 75 years of age.
4. No relevant therapy prior to surgery.

The primary tumour was treated with breast-conserving surgery or modified radical mastectomy with axillary lymph node dissection in the years 1985–1987. Patients without axillary involvement (N-) had no further therapy. The rest of the cases (N+) were treated with six courses of adjuvant chemotherapy [most commonly cytoxan, methotrexate, 5-fluorouracil and Adriamycin (doxorubicin)] in premenopausal women and tamoxifen 20 mg/day for 3 years in postmenopausal women. A follow-up time of at least 84 months was available for all patients.

Tumour samples

Twenty-nine tumours (46%) were located in the upper outer quadrant, 9 (14.3%) in the upper inner quadrant, 6 (9.5%) in the lower outer quadrant, 3 (4.8%) in the lower inner quadrant, 11 (17.5%) centrally within 1 cm of the areola, and 5 (7.9%) were multifocal. Multicentricity (as defined by cancer presence in a quadrant other than the one containing the dominant mass) was detected in three cancers. ER and progesterone receptors (PgR) were measured using the dextran-coated charcoal (DCC) ligand binding biochemical assay. The level of 10 fmol/mg of cytosolic protein was used throughout to categorise both ER and PgR as positive or negative. Tumour size was tabulated according to the recommendations of the International Union Against Cancer. Small neoplasms (≤2 cm) were regarded as pT1 and were segregated from all other lesions regardless of size (pT2, i.e. diameter of more than 2 cm but less than 5 cm as far as our specimens are concerned), yielding two clusters.

Histopathology

All selected neoplasms belonged to the classic (NOS) group and their grading was performed according to Bloom and Richardson's principles [1]. Areas of necrosis, , usually of a high histological grade, occurred in 31 samples (49.2%). A mononuclear inflammatory infiltrate at the interphase between tumour and stroma was present in 38 cancers (60.3%). Tumour and/or dermal lymphatic emboli and marked, Alcian blue–PAS stained, extracellular mucin were present in 17 (27%) and 4 (6.3%) tumours respectively. Features suggestive of endocrine differentiation were absent.

Immunohistochemistry

A three-step immunoperoxidase staining technique was used on paraffin-embedded 4-μm-thick tissue sections from primary tumours and adjoining uninvolved surrounding tissue. After deparaffinisation through graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 0.1% hydrogen peroxide in methanol for 20 min. Immunostaining was performed using the strept-ABC/complex peroxidase method (Dako, Denmark). No enzymatic digestion was carried out prior to incubation with antibodies. As primary antibodies (Ab) we used the monoclonal M1G8 Cat D antiserum (dilution 1:30), the monoclonal G3.1 Hsp 27 Ab (dilution 1:350) and the pS2 Ab (dilution 1:100) (Biogenex), which recognises native pS2 protein and is not known to cross-react with other molecules such as epidermal growth factor (EGF) or tumour necrosis factor α. Diaminobenzidine tetrahydrochloride 0.5 mg/ml in phosphate-buffered saline containing 0.03% hydrogen peroxide was used as a chromogen.

Tumour sections subjected to the whole procedure except for incubation with the primary Ab were used as substitute "negative" controls. Sections of MCF-7 cells suspended in 2% agar, fixed in 10% buffered formalin for 24 h and embedded in paraffin were used as positive controls for Cat D and Hsp 27 immunopositivity. Previously tested breast cancer tissue sections positive for pS2 gene expression, as evidenced by DNA sequencing, were used as positive controls.

Scoring

All immunostained slides were analysed and scored in a blinded fashion by two different observers with a high degree of consistency between them (90%). In each section, at least 15 high-power fields (×400) were examined under light microscopy and the mean proportions of Cat D-positive, pS2-positive and Hsp 27-positive neoplastic cells among all malignant cells were separately calculated by cell counts. Staining intensity was also taken into account. As in previous studies, such as that of Isola et al. [13], the optimal cut-off point for Cat D immunostaining was the 10th percentile of strongly positive neoplastic cells among all tumour cells examined. Staining of in situ components, stromal cells or macrophages was not considered in the scoring but was evaluated as present or absent. With regard to pS2 and Hsp 27 immunostaining interpretation, if more than 1% of invasive tumour cells were positive, the tumour was considered positive, in line with the policy of Koerner et al. [16] and Thor et al. [29] respectively.

Statistical methods

Statistical analysis was carried out with Fisher's exact test and with the linear trend in proportions.

Results

Cat D-positive immunolabelling was detected in cancer cells of 23 tumours (37%), and marked intratumour heterogeneity was frequently observed, with an occasional topographical preference for the periphery of the tumour. Cat D immunostaining was localised in coarse or tiny intracytoplasmic granules in the cell periphery (Fig. 1), an observation compatible with the lysosomal localisation of the detected antigen. In a significant proportion of Cat D-positive neoplasms (n = 7; 30.4%), tumour-infiltrating, morphologically distinct macrophages were also Cat D-positive and so served as an internal positive control. Immunopositive macrophage-like cells, surrounding clumps of tumour cells, were also found in some cancers that lacked immunopositive malignant cells (n = 7). Apart from macrophages, some stromal fibroblasts and normal breast epithelium demonstrated focal Cat D immunoreactivity. The expression of the enzyme in tumour-adjacent in situ carcinomas was relatively low. The only significantt statistical association was observed between positive Cat D immunostaining and the involvement of

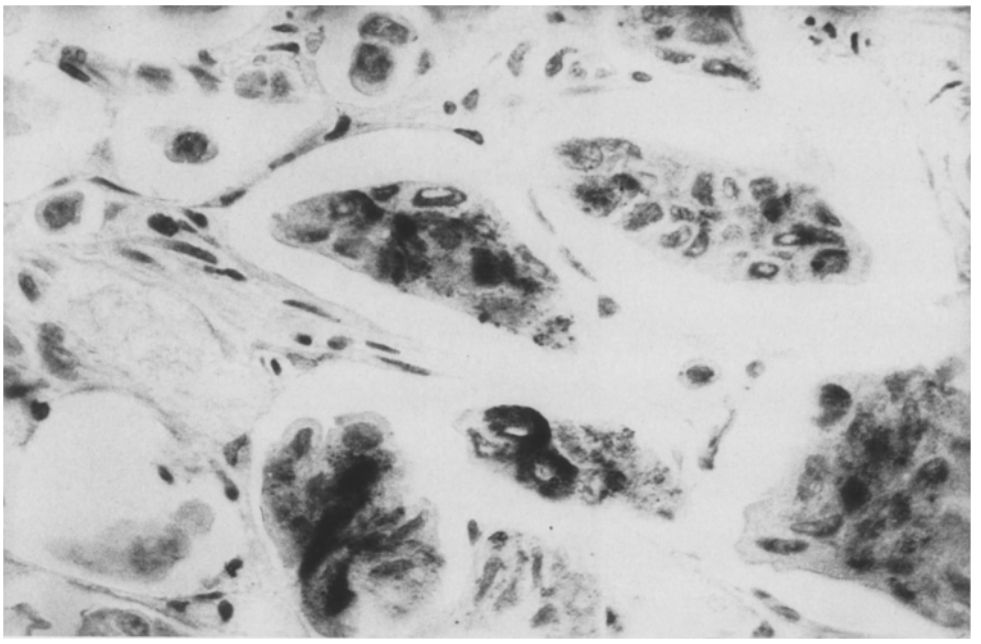

Fig. 1 Fine granular cathepsin D immunoreactivity in malignant cells (ABC-HRP, ×300)

Table 1 Correlation between Cat D expression and variables examined

Variables	*n*	Cat D positive	*P*
Menopausal status (age) at diagnosis			
<50 years	23	10 (43.5%)	P_f=0.27
≥50 years	40	13 (32.5%)	
Tumour size			
pT_1 (≤2 cm)	9	3 (33.3%)	P_f=0.57
pT_2 (>2 cm)	54	20 (37.0%)	
Oestrogen receptor status			
<10 fmol/ml	15	4 (26.7%)	P_f=0.28
10–100 fmol/ml	33	13 (39.4%)	P_t=0.45
>100 fmol/ml	15	6 (40.0%)	
Progesterone receptor status			
<10 fmol/ml	17	7 (41.2%)	P_f=0.43
10–100 fmol/ml	31	8 (25.8%)	P_t=0.53
>100 fmol/ml	15	8 (53.3%)	
Grade			
G1	8	2 (25%)	P_f=0.38
G2	42	17 (40.5%)	P_t=0.94
G3	13	4 (30.8%)	
Number of infiltrated lymph nodes			
0	26	3 (11.5%)	P_f=0.0005
1–4	14	12 (85.7%)	P_t=0.07
	}37	}20 (54%)	
>4	23	8 (34.8%)	
Overall survival			
≤36 months	13	6 (46.2%)	P_f=0.31
36–60 months	11	4 (36.4%)	P_t=0.42
≥60 months	39	13 (33.3%)	
Hsp 27 status			
Positive	39	15 (38.5%)	P_f=0.45
Negative	24	8 (33.3%)	

Table 2 Correlation between pS2 expression and variables assessed

Variables	*n*	pS2 positive	*P*
Menopausal status (age) at diagnosis			
<50 years	23	12 (52.2%)	P_f=0.19
≥50 years	40	15 (37.5%)	
Tumour size			
pT_1 (≤2 cm)	9	5 (56.0%)	P_f=0.32
pT_2 (>2 cm)	54	22 (40.7%)	
Oestrogen receptor status			
<10 fmol/ml	15	1 (7.0%)	P_f=0.0009
10–100 fmol/ml	33	19 (57.6%)	P_t=0.03
>100 fmol/ml	15	7 (46.7%)	
Progesterone receptor status			
<10 fmol/ml	17	4 (23.5%)	P_f=0.05
10–100 fmol/ml	31	17 (54.8%)	P_t=0.31
>100 fmol/ml	15	6 (40.0%)	
Grade			
G1	8	6 (75.0%)	P_f=0.06
G2	42	17 (40.5%)	P_t=0.07
G3	13	4 (30.8%)	
Number of infiltrated lymph nodes			
0	26	10 (38.5%)	P_f=0.37
1–4	14	6 (42.9%)	P_t=0.51
	}37	}17 (45.9%)	
>4	23	11 (47.8%)	
Overall survival			
≤36 months	13	6 (46.2%)	P_f=0.51
36–60 months	11	5 (45.5%)	P_t=0.72
≥60 months	39	16 (41.0%)	
Cat D immunoreactivity			
Positive	23	13 (56.5%)	P_f=0.08
Negative	40	14 (35.0%)	

axillary nodes with tumour (P_f = 0.0005, Table 1). When the number of infiltrated lymph nodes was taken into account, this tendency did not achieve statistical significance (P_t = 0.07). Except for a statistically nonsignificant association with pS2 immunoreactivity (P_t = 0.08, Table 2), no correlations were found between Cat D immunostaining and survival, ER presence or any other variable assessed (Table 1).

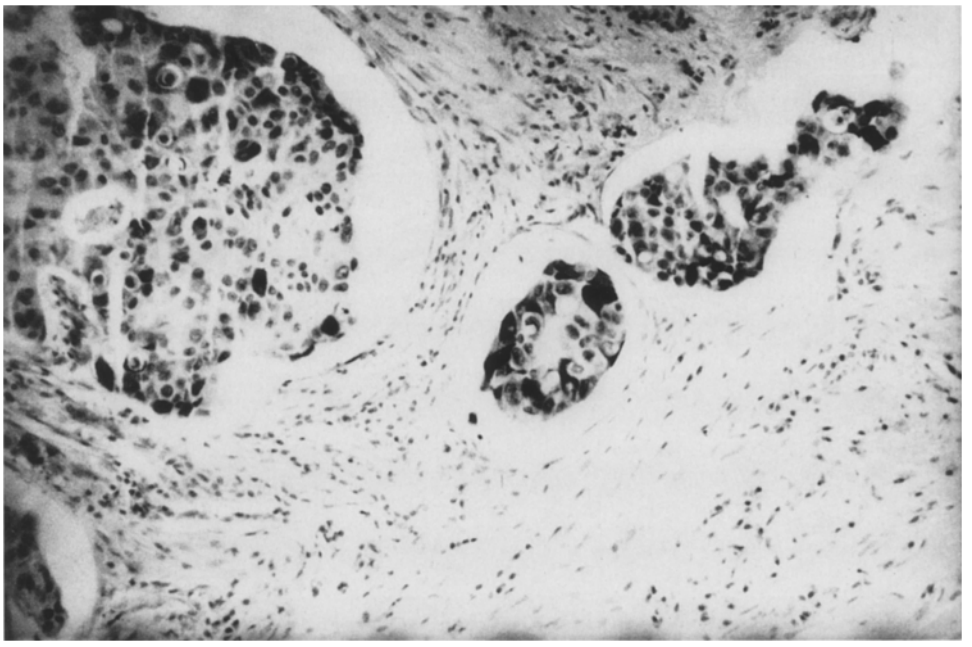

Fig. 2 pS2 cytoplasmic immunodetection in many cancerous cells (ABC-HRP, ×150)

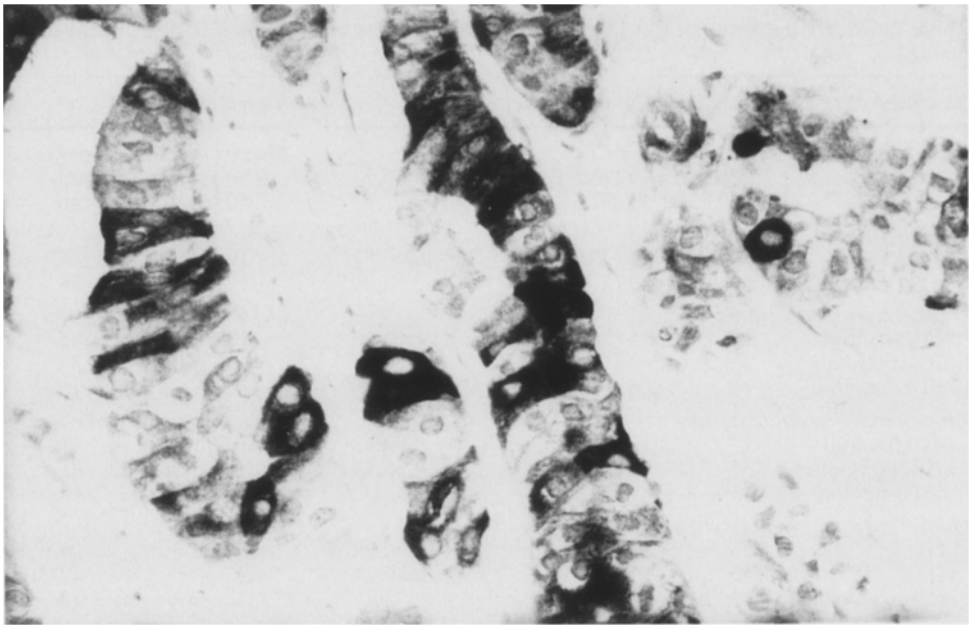

Fig. 3 pS2 immunopositivity in cytoplasm of many breast cancer cells (ABC-HRP, ×300)

pS2 protein was detected in 27 carcinomas (42.9%), where it showed diffuse cytoplasmic staining as a rule (Figs. 2, 3). Notably, all neoplasms with increased extracellular mucin synthesis (n = 4) demonstrated high pS2 immunoreactivity rates. In general, there was great heterogeneity in the distribution of pS2 immunostaining amongst tumours and within the same tumour, where intensity of immunostaining varied from cell to cell. The main cellular staining pattern was cytoplasmic with occasional perinuclear reinforcement. Stromal cells, inflammatory cells, myoepithelial cells and blood vessels were not stained. Adjacent normal breast tissue was generally negative, whereas adjacent intraductal carcinomas were frequently pS2 positive.

The presence of pS2 peptide was associated significantly with ER presence (P_f = 0.0009, P_t = 0.03, Table 2) and with PgR positivity (>10 fmol/mg protein) (P_f = 0.05) and nonsignificantly with low tumour grade (P_f = 0.06, P_t = 0.07).

Regardless of the proportion of positive cells, Hsp 27 was expressed in the cytoplasm of 39 (61.9%) cases. More than 10% of cells were positive in 15 instances (38.5%). The staining was cytoplasmic and diffuse in all cases (Figs. 4, 5). Low Hsp 27 immunoexpression was observed in tumour-adjacent, ductal cells of some lobules, which sometimes demonstrated features of apocrine metaplasia. A significant relationship was observed between Hsp 27 expression and the number of infiltrated

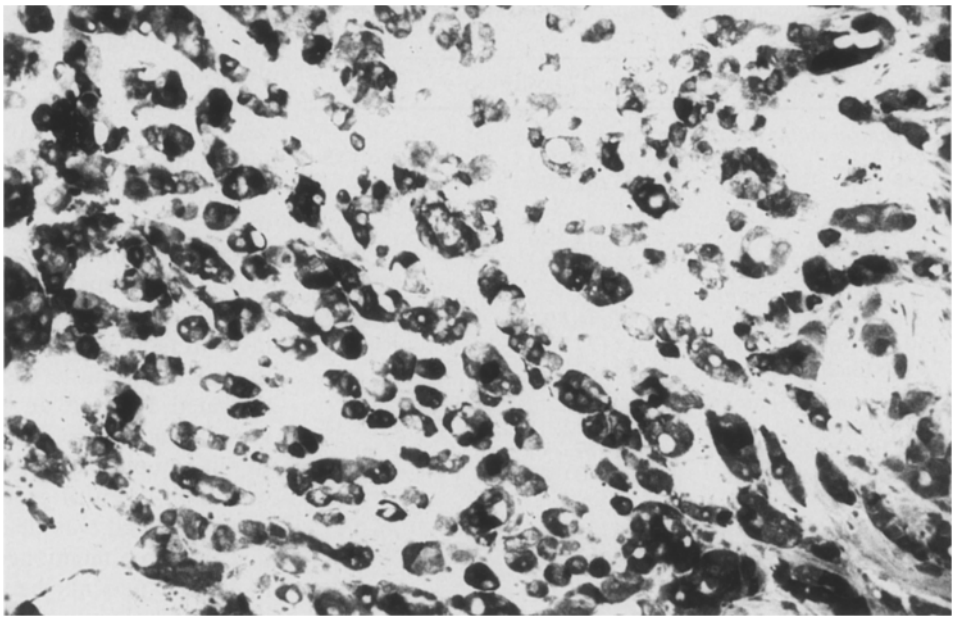

Fig. 4 Intense Hsp 27 immunolabelling in a ductal invasive carcinoma (ABC-HRP, ×150)

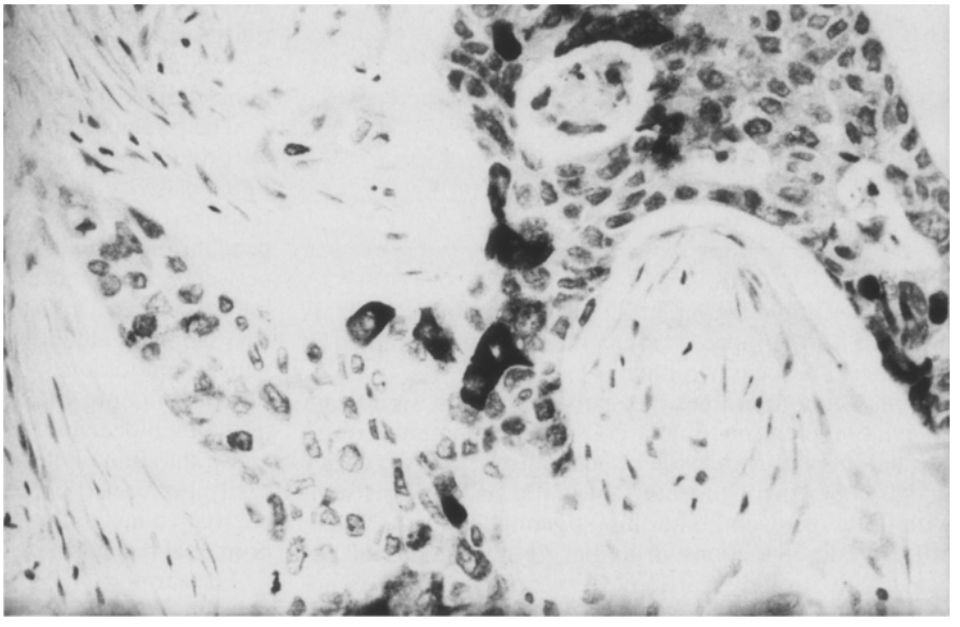

Fig. 5 Few Hsp 27-positive neoplastic cells in a ductal invasive carcinoma (ABC-HRP, ×300)

lymph nodes (P_t = 0.05, Table 3). Hsp 27 immunoreactivity was related to the subgroup of 14 patients with one to four metastatically infiltrated lymph nodes. Despite chemotherapy, 10 of the 12 Hsp 27-positive patients of this subgroup died within 5 years of surgery, and thus even the weak, nonsignificant association (P_t = 0.11, Table 3) between Hsp 27 immunoreactivity and short survival (<5 years) appears meaningful. No correlation between Hsp 27 and ER content or any other variable emerged from this survey.

Discussion

Cell products synthesised under oestrogen control have recently entered the field of basic research, since their potential prognostic value may identify patient subgroups needing specific therapeutic options. In principle, immunohistochemistry (IHC) provides distinct advantages over cytosolic assays in analysing oestrogen-dependent proteins in breast cancer. The latter cannot distinguish the cellular origin of the evaluated proteins and especially of Cat D, which is notably expressed in nonmalignant cells and neoplastic cells. IHC preserves morphological information, allowing separate visual analysis of Cat D expression, and Cat D overestimation in tumours

Table 3 Correlation between Hsp 27 expression and variables examined

Variables	*n*	Hsp 27 positive	*P*
Menopausal status (age) at diagnosis			
<50 years	23	14 (60.8%)	P_f=0.55
≥50 years	40	25 (62.5%)	
Tumour size			
pT_1 (≤2 cm)	9	4 (44.4%)	P_f=0.14
pT_2 (>2 cm)	54	35 (64.8%)	
Oestrogen receptor status			
<10 fmol/ml	15	7 (46.7%)	P_f=0.027
10–100 fmol/ml	33	24 (72.7%)	P_t=0.71
>100 fmol/ml	15	8 (53.3%)	
Progesterone receptor status			
<10 fmol/ml	17	9 (52.9	P_f=0.34
10–100 fmol/ml	31	24 (77.4%)	P_t=0.52
>100 fmol/ml	15	6 (40.0%)	
Grade			
G1	8	6 (75.0%)	P_f=0.57
G2	42	22 (52.4%)	P_t=0.39
G3	13	11 (84.6%)	
Number of infiltrated lymph nodes			
0	26	11 (42.3%)	P_f=0.21
1–4	14	12 (85.7%)	P_t=0.05
	}37	}28 (75.7%)	
>4	23	16 (69.6%)	
Overall survival			
≤36 months	13	10 (76.9%)	P_f=0.18
36–60 months	11	8 (72.7%)	P_t=0.11
≥60 months	39	21 (53.8%)	
pS2 status			
Positive	27	18 (66.7%)	P_f=0.34
Negative	36	21 (58.3%)	

heavily infiltrated by Cat D-positive stromal cells is avoided. Furthermore, malignant cells can be studied in their actual tissue environment.

This study reinforces the statistically most significant association between tumour cells' Cat D immunopositivity and tumour extension as demonstrated by axillary lymph nodes involvement (Table 1), a finding consistent with those of several other investigations [11, 14, 22, 26, 30]. So, serial sections in axillary lymph nodes of patients with strongly Cat D-positive primary tumours are most likely to detect micrometastases, not yet clinically evident, which might even escape routine pathological procedures. Moreover, our observation that Cat D immunostaining was less marked in tumour-adjacent, in situ carcinomas than in invasive areas further supports the hypothesis that Cat D is involved in promoting invasion of breast cancer, being the major protease secreted by neoplastic cells responsible for degradation of the extracellular matrix [11].

Although no apparent correlation between Cat D immunolabelling and poor survival emerged from this survey, it is worth noting that the three patients with axillary node-negative, Cat D-positive breast cancers were characterised by poor prognosis dying within 3 years of surgery. The unfavourable prognostic significance of Cat D detection in axillary node-negative patients [13] is worth investigating using morphological methods, since it has been suggested that high Cat D levels signify inflammatory cell involvement within the tumour, a factor paradoxically associated with poor prognosis [23]. Nevertheless, Cat D expression in the macrophages of our cases was not associated with onset of metastases or survival, in accordance with the finding of Isola et al. [13].

The lack of influence of Cat D on patient survival may be attributed to the pathway of its synthesis. In this study, as in some others [28], no correlation emerged between Cat D immunopositivity and the presence of ERs, known to characterise tumours with a prognostic advantage. In fact, at least two pathways may influence procathepsin D synthesis: a direct transcriptional regulation by activated ER and an indirect regulation via autocrine growth factors (EGF and insulin-like growth factor II). In our samples, Cat D expression is probably associated with high levels of growth factors and thus no relation to better survival was detected. Let us point out, however, that the three mentioned above of four ER-negative, Cat D-positive patients died within 3 years of surgery, implying that Cat D immunodetection in hormone-independent [ER(-)] and thus anti-oestrogen-resistant cancers is an adverse prognostic indicator [5, 22]. Cat D immunoreactivity was nonsignificantly associated with pS2 protein detection (Table 2), a finding in parallel with that of Gion et al. [10].

The pS2 gene product was the only oestrogen-regulated protein which was clearly associated with ER and PgR positivity in this study (P_f = 0.0009 and P_f = 0.05 respectively). As PgR are a well-known oestrogen-dependent marker, one should logically expect correlation between these receptors and pS2 gene expression in breast cancer. This strong correlation between pS2 and ERs is in agreement with other recent studies [9, 10, 20] which tend to conclude that pS2 expression is indicative of a functioning ER machinery, not always reliably detected by biochemical assays. So, from a clinical point of view, the immunohistochemical assay of this protein, easily performed on paraffin sections, may represent an alternative method to cytosol assays for the prediction of hormonal responsiveness of breast tumours.

Furthermore, the possible relationship between cytoplasmic pS2 expression and low tumour grade, as found in other investigations [2, 7, 20, 21], indicates not only the increased likelihood of ER detection in well-differentiated tumours but also a highly organised state of cellular physiology as far as malignant cells are concerned. Interestingly, a general trend has been reported according to which pS2 is frequently positive in tissues which differentiate into glandular structures with secretory functions [6, 17, 20]. Let us point out here that the four mucin-producing cancers of our survey demonstrated high pS2 immunopositivity. This finding supports the suggested role for pS2 in facilitating the transport or the packaging of high concentrations of mucins prior to secretion [17]. In general, well-differentiated tumours are likely to preserve the functional integrity of the biochemical pathways and synthesising systems which are responsible for pS2 expression.

The value of detecting Hsp 27 in patients with breast cancer as a predictor of response to hormonal treatment remains controversial. Our study failed to demonstrate a statistical correlation between Hsp 27 immunoreactivity and ER presence, although a significant association was found between Hsp 27 immunodetection and the finding of one to four nodal metastases. This may explain the weak association of this marker with poor overall survival of our patients. It has been postulated that Hsp 27 may participate along with the larger Hsp [7] in the development of resistance to various stressful conditions and agents, including cytotoxic drugs [30], in particular doxorubicin [3, 19], a drug used in some of our patients. This may explain the short survival of the 10 of 12 Hsp 27-positive patients with one to four infiltrated lymph nodes. This resistance phenomenon associated with Hsp may be permanent (overexpression due to genetic alterations) or transient; it seems to be selective for certain drugs, and it may be triggered not only by heat shock but also by other stresses that usually occur in human tumours.

In conclusion, we have confirmed that Cat D is a protein clearly associated with tumour invasiveness and the presence of lymph node metastases and that pS2 protein expression is under the direct influence of oestrogens. Hsp 27 appear to be connected with the presence of one to four nodal metastases. Thus, interesting implications concerning patient survival have emerged for all three markers. Further study of larger samples is needed to clarify the exact prognostic significance of each protein in specific subgroups of patients.

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