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Acknowledgements—We thank Herbie Newell for help in reviewing the paper. This work was supported by a grant of the Ligue contre le Cancer du Rhône.

Eur J Cancer, Vol. 28A, No. 8/9, pp. 1315–1318, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Prognostic Relevance of pS2 Status in Association With Steroid Receptor Status and Proliferative Activity in Node-negative Breast Cancer

V. Cappelletti, D. Coradini, E. Scanziani, E. Benini, R. Silvestrini and G. Di Fronzo

Expression of the oestrogen-regulated pS2 protein was investigated on paraffin-embedded sections of primary breast tumours from 200 node-negative patients. Immunoreactivity was observed in 56% of the cases. pS2 expression was inversely correlated with tumour size and proliferative activity, whereas a direct correlation was observed with steroid receptor. 5-year relapse free survival was influenced by tumour size ($P = 0.02$), oestrogen receptor status ($P < 0.05$), and proliferative activity ($P < 0.01$). No difference in relapse-free survival was observed between patients subdivided according to pS2 expression alone. However, among patients with oestrogen-receptor-negative tumors, pS2 expression predicted a shorter relapse-free survival.
Eur J Cancer, Vol. 28A, No. 8/9, pp. 1315–1318, 1992.

INTRODUCTION

THE pS2 gene has been identified in the human hormone-responsive breast cancer cell line MCF-7 as a gene regulated by oestradiol at the transcriptional level [1]. Subsequent cloning and sequencing revealed that the pS2 gene codes for a secreted, low-molecular-weight protein similar to a porcine pancreatic protein known to inhibit gastrointestinal mobility and secretion [2]. Expression of the pS2 protein has been observed in human breast tumours, oestrogen-receptor-positive (ER+) breast cancer cell lines, and normal stomach mucosa [2]. Interestingly, pS2 could not be detected in normal breast tissue.

The pS2 gene appears to be potentially useful in basic studies to elucidate the molecular mechanism of oestrogen activity and in clinical studies for a better prognostic resolution within ER+ tumours [3]. In fact, identification of prognostic markers is particularly critical in breast cancer owing to the variable clinical outcome in patients with tumours with a similar histology or stage. Such clinical heterogeneity results from the multiple steps of malignant evolution, which are accompanied by the acquirement of genetic alterations resulting in abnormal expression of certain proteins [4, 5].

The biological prognostic indicators most widely used for

breast cancer are steroid receptor status, ER and progesterone receptors (PgR) and the proliferative activity.

The most accurate prognostic predictions are usually obtained by using the different biological variables in association [7].

In the present study we have retrospectively evaluated the prognostic relevance of the oestrogen-regulated protein pS2 alone or in association to the classical prognostic indicators ER status and proliferative activity, evaluated as [^{3}H]thymidine labelling index (^{3}H-dT LI), in node-negative breast cancer patients.

PATIENTS AND METHODS

Patients

The study was carried out on a series of 200 patients with primary resectable breast cancer who underwent radical mastectomy at the Istituto Nazionale Tumori of Milan during October 1975 to September 1983. All patients had histologically proven negative nodes (the median number of nodes examined was 14) and no evidence of distant metastases by clinical, radiological and radioisotopic examination at the time of presentation. All patients were submitted only to radical surgery as the first-line treatment. No postoperative therapy was administered until new disease manifestation was documented. Patients were examined in the institute's outpatient clinic every 6 months during the first 5 years and yearly thereafter. Disease status was assessed through physical examination, chest roentgenogram and skeletal survey. The overall relapse rate at 6 years was 40.5%. The follow-up time ranged from 4 to 114 months, with a median of 70 months.

In vitro *determinations*

Fragments sampled from the different areas of the tumour were frozen immediately after surgery and stored in liquid nitrogen for cytoplasmic ER and PgR determination by the dextran-coated charcoal technique as previously described [8], or immediately processed for the determination of proliferative activity by ^{3}H-dT incorporation. Briefly, tumour fragments of a few mm^3 were incubated for 1 h at 37°C with complete culture medium containing [^{3}H]thymidine (222 KBq/ml; specific activity 185 GBq/mmol) and submitted to the histoautoradiographic procedure as described previously [9]. The ^{3}H-dT LI was determined by scoring a total of more than 3000 cells on different specimens from each tumour and defined as the per cent ratio between labelled and total tumour cells.

pS2 expression was evaluated by immunohistochemistry on 6 μm sections obtained from the same paraffin-embedded samples used for ^{3}H-dT LI determination. The procedure involves staining with the avidin–biotin-peroxidase complex [10] using commercial immunoperoxidase kits (Vectstain Elite; Vector Laboratories). Sections were deparaffinised, rehydrated through graded alcohol, and incubated at 4°C for 18 h with a 1:700 dilution of a mouse anti-pS2 monoclonal antibody (courtesy of Dr Chambon). After washing, the sections were covered with a secondary antibody, i.e. biotinylated horse anti-mouse immunoglobulin, and left at room temperature for 30 min. Sections were incubated with diaminobenzidine–hydrogen peroxide for 7 min, washed in tap water, and counterstained with Meyer's haematoxylin. For each sample, a negative control was made by omitting the primary antibody. At least 200 tumour cells were counted from each sample. All assay results were evaluated by E. Scanziani.

Table 1. Correlation between pS2 and clinico-biological characteristics

	No. of cases	pS2 positive (%)	pS2 negative (%)	*P* value
Age (years)				
≤50	133	56	44	NS
>50	67	55	45	
Menopausal status				
pre+para	135	56	44	NS
post	65	55	45	
Tumour size (cm)				
≤2	92	67	33	<0.005
>2	102	43	57	
Oestrogen receptor				
ER+	133	71	29	<0.0001
ER−	67	27	73	
Progesterone receptor				
PgR+	40	77	23	<0.0001
PgR−	44	23	77	
^{3}H-dT LI				
Low	99	68	32	<0.001
High	101	45	55	

NS = not significant.

Data analysis

Tumours containing more than 10 fmoles of ER per mg of protein or more than 25 fmoles of PgR per mg of protein were considered ER+ or PgR+, respectively. Tumours were defined as slowly or rapidly proliferating by using the ^{3}H-dT LI cutoff of 2.8%, which corresponded with the median value and proved to be prognostically significant in a previous study [9]. With regard to pS2 expression, tumours containing less than 5% stained cells were considered as negative.

The association between the expression of pS2 and the different biological and clinico-pathological variables was assessed by the χ^2 test. Relapse-free survival (RFS) was computed starting from the date of surgery by means of the Kaplan–Meier product-limit method [11]. The log-rank test was used to assess differences among subgroups [12].

RESULTS

Patient and tumour characteristics are reported in Table 1. Most of the patients were under 50 years of age and premenopausal. ER were present in 66% of the tumours. Tumours greater or less than 2 cm in their largest diameter and tumours rapidly or slowly proliferating were equally represented.

A wide intertumour variability of the percentage of pS2-positive cells as well as intratumour differences in cell staining intensity was observed (Fig. 1). Immunoreactivity for pS2 was exclusively detected in the cytoplasm. In a few cases, secretory material present in the lumen of tumour-cell-composed tubular structures was positive. The distribution pattern of pS2

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Revised 11 Feb. 1992; accepted 2 Mar. 1992.

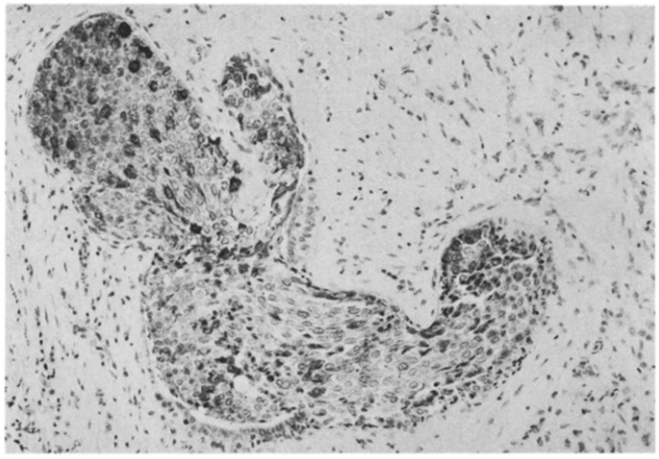

Fig. 1. Lobular infiltrating carcinoma. Immunoperoxidase for pS2 protein, haematoxylin counterstain, 350 ×. Moderate to weak positivity is detectable in the cytoplasm of most of the tumour cells. Strong immunoreactivity is evident in some cells.

expression is shown in Fig. 2. 56% of the tumours were defined as pS2+.

The association between pS2 positivity and other clinico-pathological characteristics is reported in Table 1. There was no significant association between pS2 expression and age or menopausal status. Conversely, tumour size was significantly correlated with pS2 expression. In fact, more small tumours were pS2 positive than large tumours. Moreover, a strong correlation was observed between pS2 expression and steroid receptor status and proliferative activity. In fact, more than two-thirds of pS2-positive tumours exhibited ER and PgR or a low proliferative activity.

In this series of patients, RFS at 5 years was similar in different age subgroups, whereas it was significantly influenced by tumour size, ER status and cell kinetics (Table 2). In patients with tumours smaller than 2 cm, RFS was 72% compared with 57% observed for patients with tumours larger than 2 cm ($P = 0.02$). In patients with ER+ tumours, RFS was 68% compared with 57% for patients with ER− tumours ($P < 0.05$). Patients with slowly proliferating tumours were characterised by a better RFS than patients who presented with rapidly proliferating cancers (74% vs. 54%; $P < 0.001$). Conversely, no difference in RFS was observed for patients with pS2+ or pS2− tumours when the overall series (59% vs. 60%) or sub-

Table 2. Prognostic relevance of clinico-pathological and biological factors: univariate analysis

	5 years RFS %	*P* value
Age (years)		
≤50	68	NS
>50	55	
Tumour size (cm)		
≤2	72	0.02
>2	57	
Oestrogen receptor		
ER+	68	<0.05
ER−	57	
Progesterone receptor		
PgR+	57	NS
PgR−	50	
^{3}H-dT LI		
Low	74	<0.0001
High	54	
pS2		
pS2+	59	NS
pS2−	60	

NS = not significant.

groups defined according to patient's age or tumour size were considered. However, the simultaneous consideration of ER and pS2 status showed that the lowest probability of RFS (50%) was observed for the 18 patients with ER− tumours, which also expressed pS2 (Fig. 3). Of these tumours 14 showed a high proliferative activity. A similar analysis was performed by considering pS2 status and cell kinetics (Fig. 4). RFS was mainly influenced by the proliferative activity, whereas pS2 status did not add prognostic information.

DISCUSSION

Our study confirms the results obtained by other authors about the correlation between the pS2 and steroid receptor expression. In fact, a strong association between ER or PgR and pS2 expression has been reported by using the immunohistochemical method on paraffin-embedded sections and the northern blotting procedure [13]. Similar results were also obtained

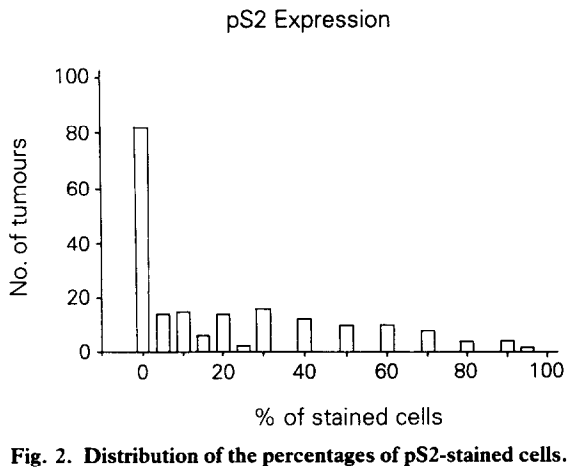

Fig. 2. Distribution of the percentages of pS2-stained cells.

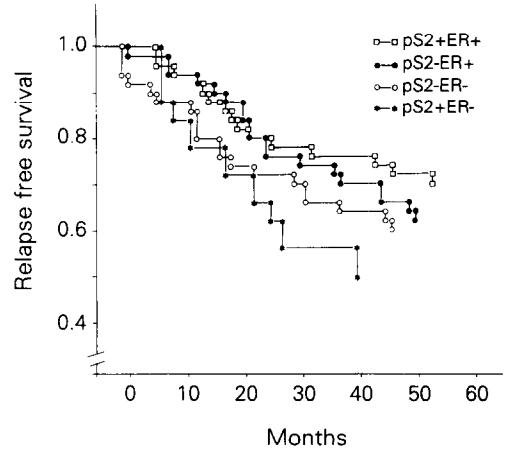

Fig. 3. Disease-free survival in node negative breast cancer patients as a function of pS2 and ER status.

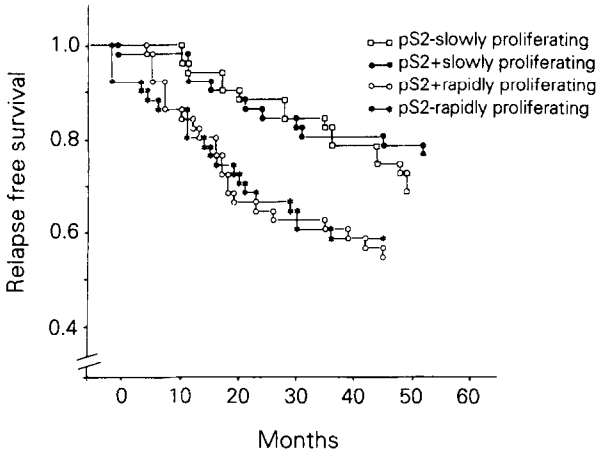

Fig. 4. Disease-free survival in node negative breast cancer patients as a function of pS2 status and proliferative activity.

by Foekens *et al.* [14] by using an immunochemical assay on tumour cytosol. Moreover, we observed an inverse relation between pS2 expression and proliferative activity, which has never been reported for human breast tumours.

However, the outcome of this study is in contrast with findings by other authors about the prognostic relevance of pS2. In fact, in the study by Abbondanzo *et al.* [15] pS2 was found to be predictive of overall survival by univariate analysis but its statistical significance was lost in the multiple regression model including other variables (age, tumour size, ploidy, ER, PgR). According to these authors, pS2 is not an independent indicator of survival and its relevance to predict RFS is limited to the ER+ PgR+ tumour subgroup. The study by Foekens *et al.* [14] appears to be more positive about the prognostic relevance of pS2. However, the study dealt with a small and heterogeneous series of N− or N+ tumours and the median follow-up was shorter than the final observation time. Moreover, it cannot be excluded that discrepancies between the results from our study and that of Foekens *et al.* are due to the different methods employed for pS2 determination. In this regard it would be interesting to compare the prognostic relevance of pS2 evaluated by immunohistochemistry and by immunochemical assay in the same series of patients.

In our hands, pS2 expression did not predict RFS and it did not add any prognostic information in the two cell kinetic subgroups. In contrast, pS2 expression allowed a prognostic discrimination within ER− tumours, but not within ER+ tumours. Interestingly, in this patient series, women with ER−, pS2+ tumours had a very high probability to relapse. This finding is in contrast with results reported in the literature, which show a prognostic advantage for patients with pS2+ tumours, and with that which could be expected from an oestrogen-regulated protein. However, pS2 was initially characterised as a product whose transcription was under oestrogenic control, but it was later demonstrated that growth factors such as EGF and IGF-I were also able to induce pS2 transcription [16]. Therefore, in ER−, pS2+ tumours an increase in pS2 expression caused by growth factors could occur, thus determining the poor clinical outcome. Furthermore, it is interesting to note that the pS2 protein has structural features similar to some small protein growth factors and shows close homology to a pancreatic spasmolytic polypeptide, which has been shown to have growth stimulatory effects on MCF-7 breast cancer cells in cultures [17]. This hypothesis is indirectly supported by the fact that more than two-thirds of our ER−, pS2+ tumours were rapidly proliferating.

However, few studies have been directed to evaluate the prognostic significance of pS2, and available information is too scanty to allow definitive conclusions. Our results are very preliminary but represent a challenge to the idea of pS2 as a favourable prognostic marker in breast cancer. Of course basic investigations are needed to clarify this issue.

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