Immunohistochemical and biochemical analysis of the oestrogen regulated protein pS2, and its relation with oestrogen receptor and progesterone receptor in breast cancer

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Abstract

Aims—(i) To assess the validity of an immunocytochemical technique for detecting pS2 protein in paraffin wax embedded tissue; (ii) to provide further data on the relation between pS2 protein and oestrogen receptor (ER) and progesterone receptor (PgR).

Methods—Breast cancer excision biopsy specimens were obtained from 35 previously untreated patients. An immunoradiometric assay was compared with an immunohistochemical method for measuring pS2 protein. ER and PgR were measured in cytosol fractions by enzyme immunoassay and the relation between the presence of these receptors and pS2 protein was assessed before and after subdivision of the women into groups of over or under 50 years of age.

Results—A good correlation was seen between the immunoradiometric and immunohistochemical methods for pS2 protein measurement ($r = 0.84$; $p = 0.0001$). Log-transformed data showed a significant correlation between increasing values of ER and pS2 protein ($r = 0.45$; $p = 0.006$) and to a lesser extent between pS2 protein and PgR ($r = 0.38$; $p = 0.03$). Correlations were also shown between pS2+ and PgR+ status ($p = 0.01$), and between ER and PgR positivity ($p = 0.05$; Fisher's exact test). pS2+ protein status was only associated with ER+ status in patients aged 50 years or less.

Conclusions—The two methods for pS2 analysis are virtually interchangeable. This provides strong support for using immunohistochemistry for pS2 in paraffin wax embedded tissue. The association with ER positivity and pS2+ protein status only in the premenopausal patients may be due to the higher levels of oestrogenic stimuli in that group.

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The value of assessing hormone receptors in the clinical management of breast cancer is somewhat equivocal.¹⁻³ Nevertheless, to enhance the understanding of the epidemiology and biology of breast cancer and the normal breast,⁴ the availability of accurate methods to measure hormone receptors and hormone-regulated proteins is important and the methodologies must be well validated.

The availability of monoclonal antibodies

raised specifically against human oestrogen receptor (ER),⁵ progesterone receptor (PgR),⁶ and pS2 protein⁷ has allowed the expression of receptors in breast cancer to be measured by immunochemical means using extracts prepared from the cytosols of tumour biopsy specimens. Enzyme immunoassay (EIA) is beginning to replace the radioligand binding assay with dextran-coated charcoal (DCC) for steroid receptors because of the improvements in reproducibility between and within laboratories.^{8,9} Immunohistochemical analyses of ER and PgR in frozen tissue sections and, more recently, paraffin wax embedded tissues have also proved viable alternatives to DCC² and EIA.¹⁰ The measurement of PgR is usually on the premise that this steroid receptor is regulated by oestrogen and will only be expressed in tissues in which the oestrogen regulatory system through ER is intact.¹¹⁻¹³ This might then be expected to provide a more accurate indicator of the oestrogen dependence of individual breast carcinomas. It has become clear, however, that while ER positive and PgR positive tumours are likely to respond to oestrogen deprivation, these steroid receptors are far from perfect as response predictors. It has been suggested that the more recently described product of the oestrogen regulated gene pS2 might be a better predictor.¹⁴⁻¹⁶ Good correlations have been shown between pS2 protein measured by immunoradiometric assay (IRMA) and northern blotting for determining pS2-mRNA.¹⁷ The expression of this protein may also be examined by immunohistochemical analysis (IHA).¹⁸ It is important to determine the comparability of the two methods as, in prognostic and biological studies of breast cancer, biopsy specimens are frequently too small for IRMA.

Methods

The biopsy specimens were sent to the histopathology department immediately after resection for biochemical and histological analysis. The tissue for biochemical analyses was snap frozen in liquid nitrogen immediately after dissection from the breast. The specimens were stored at -80°C and analysed for ER in batches within two weeks of sampling. PgR and pS2 were analysed in a single batch.

Samples were obtained from 35 patients who had been diagnosed with primary operable breast cancer. The data were analysed separately for patients above or below 50

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years of age (range 35–77 years; median 53 years). This age subdivision was found to be analogous to menopausal status with three exceptions. The size of each tumour was estimated as a product of two diameters noted at pathological examination. The lymph node status was available in 25 patients and was defined by the presence or absence of malignant tumour in the excised nodes. Tumour differentiation was noted from the histopathological records. The distribution of these demographic data is summarised in table 1.

IMMUNOHISTOCHEMICAL METHOD FOR PS2 AND SCORING

Portions of tissue taken from the breast biopsy specimen at surgery were processed for routine histopathological analysis by fixation in 10% neutral buffered formalin (pH 7.4) for 24 hours and paraffin wax embedding at 56°C. The wax sections were cut at 3 µm on to poly-L-lysine-coated microscope slides and were air dried overnight in an oven at 37°C. Dewaxing in xylene and rehydration through a descending series of alcohols was followed by blocking endogenous peroxidase activity with 10% hydrogen peroxide in phosphate buffered saline (PBS) (pH 7.4) for 15 minutes. After washing the sections in running tap water for 5 minutes they were rinsed in PBS plus 1% Tween 80. The PBS was decanted from the sections and any excess fluid wiped off before incubating in mouse anti-pS2 monoclonal antibody at a dilution of 1 in 400 for 2 hours. The washing and decanting procedure was repeated and the sections were incubated in biotinylated rabbit anti-mouse immunoglobulin (Dako) for 45 minutes at a dilution of 1 in 200. After the washing procedure, the tissues were incubated in pre-prepared streptavidin-biotinylated complex and horseradish peroxidase for 30 minutes and washed again. The peroxidase activity was detected using 1% diaminobenzidine (free base) dissolved in dimethyl formamide plus 100 µl of 30 volumes hydrogen peroxide per 100 ml for 10 minutes. The sections were then washed in running tap water for 5 minutes, counterstained lightly with Mayer's haematoxylin for 30 seconds, blued in tap water, dehydrated in graded alcohols, cleared in xylene and mounted in Depex. Negative control slides were prepared from samples known to be pS2 positive by exclusion of the primary antibody.

Scoring was assessed without prior knowledge of the results, by counting the number of malignant cells staining and expressing this as a percentage of the total number of malignant cells. A tumour was designated pS2 positive if 10% or more of malignant cells stained.

IMMUNOASSAY FOR ER, PgR, AND PS2

Before cytosol preparation, fat, blood, and necrotic tissue were discarded from each sample and a piece of tumour between 0.1–0.2 g was taken. The tissue was finely chopped, weighed, and then transferred to the Teflon container of a microdismembrator (Braun Medical Ltd, Aylesbury, Buckinghamshire, England). The tissue was pulverised for 1 minute after cooling in liquid nitrogen for 1 minute. The powdered tumour was reconstituted in iced TRIS-molybdate buffer (5 mM sodium molybdate, 10 mM monothioglycerol, 1 mM dipotassium chloride EDTA, 3 mM sodium azide and 10 mM TRIS (pH 7.4); 1:8 weight:volume). The suspension was then vortexed, the ball bearing removed, and the homogenate centrifuged at 4°C for 20 minutes at 2000 × *g*. The homogenate was kept cool while the cytosol fraction was removed and diluted 1 in 5 in TRIS-molybdate buffer. This was then assayed for protein using the Bio-Rad assay with a bovine gamma globulin standard. An aliquot of the cytosol was then adjusted to give a protein concentration of 1 to 2 mg/ml.

The oestrogen and progesterone receptor contents in the diluted cytosols were determined using the ER-EIA and PgR-EIA kits from Abbott Diagnostics (Chicago, Illinois, USA) according to the manufacturer's instructions and values of ≥ 10 fmol/mg protein and ≥ 15 fmol/mg protein, respectively, were regarded as positive. The pS2 protein was measured by IRMA using the Cis Kit (High Wycombe, Buckinghamshire, England). This assay uses two anti-pS2 monoclonal antibodies: the first bound to the base of a test tube and the second with an I¹²⁵ label in the liquid phase. Diluted cytosol was added to the tubes and incubated with the I¹²⁵-labelled antibody for 1 hour at room temperature. The unbound I¹²⁵-antibody was then removed by washing with distilled water. The bound counts were used to calculate the amount of pS2 protein in the cytosol.

The geometric means, regression coefficients, and regression lines were calculated using Statview and Cricket graph on an Apple Macintosh SE computer. Because of the relatively small number of patients, Fisher's exact test was applied to test for an association between the incidence of ER or PgR expression in tumours which were pS2 protein positive. The Mann-Whitney U test was applied to the ranked frequencies of combined ER/PgR *v* pS2 status. The level of significance was set at *p* < 0.05. All reported *p* values were two-sided.

Table 1 A summary of tumour size, histopathological differentiation and lymph node status in the defined patient age groups

Patient group	Mean age (years)	Tumour size*	Well diff.	Mod. diff.	Poor diff.	LN+ %	LN- %
All	53	6.1	3	11	21	56	44
≤ 50	41	4.5	9%	31%	60%	30	70
> 51	63	7.4	2	5	8	53	47
			14%	33%	53%		
			1	6	13		
			5%	30%	65%		

*Tumour size determined by the product of two diameters in units cm²
 Diff. = differentiated
 One peri-menopausal patient included in ≤ 50 years age group and two perimenopausal patients were included in > 50 years age group
 LN+ histopathological evidence of cancer in resected lymph node
 LN- no histopathological evidence of cancer in resected lymph node

Results

A summary of tumour size and differentiation

Figure 1 Photomicrograph showing immunohistochemical staining of pS2 protein in the cytoplasm of a paraffin embedded section of human breast carcinoma infiltrating cells. The normal duct is negative.

Figure 2 Simple linear regression relationship between the log transformation of the immunoradiometric (IRMA) and immunohistochemical method of measuring pS2. The regression line was fitted from the results from all patients. Combined data: $y = 0.37 + 0.87x$; $r = 0.84$; $n = 30$; $p = 0.0001$. Zero results of % pS2 positive malignant breast cells were assigned the value 0.2 and <1% positive cells were assigned the value 0.9.

and the patient's lymph node status is shown in table 1. The incidence of positive lymph nodes was 23% greater in the older age group and the tumours from the younger patients were smaller, but these differences were not significant. There was no significant relation between pS2 values and the patient's age, tumour size (linear regression analysis), or extent of tumour differentiation (Fisher's exact test). However, positive lymph node status was associated with negative pS2 protein status, measured by IRMA (Fisher's exact probability = 0.03).

Table 2 A comparison of the log transformed linear regression coefficients of the tumour samples from the patients in the defined age groups

Axis	X	All			≤50			>50		
		r	p	n	r	p	n	r	p	n
ln(pS2)	ln(ER)	0.45	0.006	35	0.79	0.0001	15	0.22	ns	20
ln(pS2)	ln(PgR)	0.38	0.03	31	0.43	ns	13	0.38	ns	18
ln(IHA)	ln(pS2)	0.84	0.0001	30	0.89	0.0001	14	0.80	0.0001	16

r = regression coefficient
ns = not significant
n = number of patients
p = probability

The surgical samples provided a large cross-section for assessment. The pS2 protein staining was confined to the cytoplasm of the breast cancer cells, with the infiltrating tumour staining principally (fig 1). The staining was granular in appearance and sometimes varied in intensity. The luminal contents of the breast ducts stained strongly and occasionally benign and normal elements of the tissue were also positive. The stroma did not stain and there was negligible background staining.

Table 2 and fig 2 show the excellent correlation between the IRMA and IHA ($r = 0.84$). A comparison of the IRMA and IHA methods by applying the Fisher exact test to the observed frequencies of the pS2 positive and pS2 negative protein status in all the patients' tumour samples further confirmed the highly significant association between these two methods ($p = 0.0001$). Seventeen out of 30 tumours were classified as immunohistochemically negative. Within these 17 pS2 negative tumours, some staining was observed in six cases (35%), but three scored less than 1%; two scored 1%, and one scored 4%.

All the tumours which were IRMA negative were also IHA negative. However, three of 16 (19%) tumours which were IRMA positive were IHA negative; the IRMA values for these three tumours were 0.97, 1.0, and 13 ng/ml. It is well recognised that discrepancies tend to occur between methods at the borderline level.¹⁹

There were 35 results in the biochemical assays measuring ER and pS2 protein (table 2). The numbers in the PgR group were reduced to 31 because there was insufficient cytosol to carry out the assay in four cases. The immunohistochemistry results were reduced from 35 to 30 because five of the patients' sections did not contain enough malignant cells for scoring.

The regression coefficients and probabilities resulting from the computation of simple linear regression analyses performed on the transformed values of the three markers are summarised in table 2. After log transformation of the receptor values there was a significant correlation between ln(pS2) and ln(ER) (fig 3); a relation also existed between ln(pS2)

Figure 3 Simple linear regression relationship between ln(pS2) and ln(ER). The regression line was fitted from the results from all patients. Combined data: $y = -1.10 + 0.55x$; $r = 0.45$; $n = 35$; $p = 0.006$. Zero results of ER were assigned the value 0.5 and zero results of pS2 protein were assigned the value 0.05.

Figure 4 Simple linear regression relation between $\ln(pS2)$ and $\ln(PgR)$. The regression line was fitted from the results from all patients. Combined data: $y = -0.25 + 0.34x$; $r = 0.38$; $n = 31$; $p = 0.035$. Zero results of PgR were assigned the value 0.1 and zero values of pS2 protein were assigned the value 0.05.

and $\ln(PgR)$ (fig 4), but to a less significant degree. Subdivision according to menstrual status showed a highly significant correlation between $\ln(pS2)$ and $\ln(ER)$ (table 2 and fig 3) in tumours from women of 50 or under but not over 50 years. This was not mirrored in the PgR results which were subdivided in a similar manner.

Fisher exact probabilities were calculated from the frequencies of ER or PgR positive or negative tumours with regard to their pS2 status by IRMA. ER and PgR positivity was significantly associated in the whole group. There were two tumours which were ER negative and PgR positive; these had borderline PgR values of 15 and 16 fmol/mg protein. PgR positivity was significantly associated with pS2 positivity but ER positivity was only associated with pS2 positivity in the younger group of patients. In women aged 50 years or younger, there was 100% concordance between pS2 positivity and those tumours which were ER positive and PgR positive whereas three of nine of the ER negative tumours and three of seven PgR negative tumours were pS2 positive. When considering the three proteins in combination, there was an association between ER+PgR+pS2+ and ER-PgR-pS2- phenotypes (Mann-Whitney probability = 0.03; table 3).

Discussion

The growth of many breast cancers is dependent on oestrogens. Oestrogen is known to up-regulate the expression of PgR¹¹ and the product of the pS2 gene.²⁰ The pS2 gene was discovered during a search for oestrogen regulated genes in the MCF-7 cell line derived from human breast cancer.^{21 22} The expression

of this gene has been found to be a good indicator of the response of breast cancer patients to endocrine treatment. In combination with ER and PgR levels the presence of pS2 protein has been reported as defining a group of patients with a very good overall prognosis with the three parameters conferring cumulative power.^{3 15} Therefore pS2 has potential as an important new tool as a prognostic factor and for studying oestrogenic regulation in breast cancers.

A reliable immunohistochemical method for measuring pS2 would be useful in the analysis of small samples and its validity in paraffin embedded material would provide the opportunity to examine archival material. Retrospective analyses in association with some of the large breast cancer trials conducted over recent years could provide further information about the prognostic importance of pS2 protein. The preservation of architecture of the tissue such that account can be taken of the heterogeneity of tissue staining¹⁹ and benign and normal elements are also advantages of the IHA approach.

The primary aim of this study was therefore to assess the validation of an immunocytochemical technique for detecting pS2 protein in paraffin embedded tissue. Our results have shown that there is an excellent correlation between the two methods for pS2 analysis to the extent that they are virtually interchangeable.

In comparisons of studies by different workers, the cut-off level accepted for positivity is of great importance. Our cut-off point of 0.97 ng/mg protein for pS2 IRMA was selected arbitrarily as the median value in our population. In Foekens' study,¹⁵ the cut-off 11 ng/mg protein was chosen by isotonic regression analysis as the most powerful predictor of early recurrence and death (in their population) and 27% pS2+ tumours resulted. In our study, the use of 11 ng/mg cut-off also resulted in 28% pS2+ tumours. This is despite the fact that the kit manufacturer's (CIS) standard is different from Foekens': 11 ng of the Foekens' standard is said to be equivalent to 30 ng CIS pS2 protein. We cannot explain this discrepancy but suggest that further work should be conducted to assess the comparability of the standards.

The secondary aim of this work was to provide further data on the relationship between pS2 and ER and PgR. The numbers studied cannot provide a comprehensive statement of the relationship but we were surprised to find that five tumours were ER-pS2+ which was 16% of the total sample (table 3). Based on the hypothesis that pS2 is an oestrogen regulated gene this anomaly is unexpected, but has been observed before. This is a higher frequency than recorded by Foekens¹⁵ and by others.^{23 24} If we apply an 11 ng/mg cut-off (see above) there are still three tumours which fall into the ER-PgR-pS2+ phenotype. If pS2 and PgR are entirely E-regulated we would not expect them to be expressed in ER-tumours. However, it is well recognised

Table 3 Mann-Whitney U test for all patients to test for any association between the patient's pS2 status and the frequency of the double receptor ER and PgR occurring in their tumours

All	Patients	pS2+	pS2-	Totals
ER+	PgR+	9	2	11
ER+	PgR-	3	5	8
ER-	PgR+	2	0	2
ER-	PgR-	3	7	10
Totals		17	14	31

p = <0.05
Two tailed test

that there is a small proportion of tumours which are ER–PgR+ and we confirm that in the present results. Additionally, it appears that pS2 may be present in ER-tumours. For PgR it has been suggested that this may be due to the presence of a variant constitutively active form of ER which is not detectable by conventional ligand binding assays or by the Abbott EIA and it has been postulated that this variant is due to an exon 5 deletion.²⁵

The indications are that the pS2 gene has two start sites for transcription and because a mRNA molecule corresponding to pSB1, and not pSB2 was detected in MCF-7 cells on RNA blot hybridisation analysis it was surmised that one transcriptional start site was mainly used.²⁶ Occurrence of the uncommon ER–PgR–pS2+ phenotype may therefore relate to the pSB2 site being switched on. This may explain at least some of the pS2+ tumours in the ER–PgR– phenotype. However, it is clear that PgR and pS2 are not interchangeable as markers of an intact oestrogen receptor mechanism. It has also been noted that EGF may regulate pS2 expression²⁷ and this route of stimulation may explain the other discrepancies. There were no cases of ER–PgR+pS2– expression.

The correlation between expression of pS2 and ER in the premenopausal women but not the postmenopausal females could be due to the fact that oestrogen levels are much higher in premenopausal women than in postmenopausal women and that oestrogen in postmenopausal women is not an important stimulator of pS2. ER and PgR levels were higher in postmenopausal patients whereas pS2 levels were similar in both groups. Greater frequency of ER positivity in postmenopausal women is well established. The fact that there were three ER–pS2+ patients in the premenopausal group and two ER–pS2+ in the postmenopausal group does not, however, support the hypothesis that oestrogen in postmenopausal patients is of lesser importance as a component in the stimulation of pS2 expression.

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