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The Role of Four Oestrogen-responsive Genes, pLIV1, pS2, pSYD3 and pSYD8, in Predicting Responsiveness to Endocrine Therapy in Primary Breast Cancer

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The expression of four oestrogen-responsive genes in 118 immunohistochemically defined primary breast tumours has been determined by northern analysis. While all the genes are induced by oestrogen in oestrogen receptor (ER)-positive cell lines, their behaviour is different in breast tumour biopsies. This behaviour can be divided into two groups; the first containing the genes, pLIV1 and pLIV2 (pS2), which both show a significant association with ER status ($P = 0.001$) and a corresponding inverse relationship with epidermal growth factor receptors (EGFR) ($P = 0.01$ and $P = 0.08$, respectively). In addition, the mRNA levels of both pLIV1 and pS2 are greater in ER-positive compared to ER-negative disease ($P = 0.001$). While a small number of ER-negative tumours were positive for either pLIV1 (12%) or pS2 (9%), we failed to observe co-expression of pLIV1 and pS2 in ER-negative disease. In ER-positive disease, four tumour populations were identified; ER+pLIV1−pS2−, ER+pLIV1−pS2+, ER+pLIV1+pS2− and ER+pLIV1+pS2+. Interestingly, the levels of pLIV1 and pS2 when co-expressed were significantly greater in ER+pLIV1+pS2+ tumours compared to either of the ER+pLIV1+pS2− ($P = 0.03$) or ER+pLIV1−pS2+ ($P = 0.01$) mixed phenotypes. Unlike pLIV1 and pS2, pSYD3 and pSYD8 belong to a group of genes which are expressed in the majority of tumours regardless of ER and EGFR status. However, lower pSYD8 mRNA levels were detected in moderately EGFR-positive disease ($P = 0.06$) while both pSYD3 positivity ($P = 0.01$) and mRNA levels ($P = 0.001$) were increased in highly proliferating tumours as shown by Ki67 immunostaining. These genes provide additional markers which, in conjunction with other parameters, may help to determine the likelihood of a given tumour to respond to endocrine therapy.
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INTRODUCTION

A NUMBER OF different biochemical and tumour markers are currently used to determine the type of therapy offered to breast cancer patients. One of these, the oestrogen receptor (ER) is particularly useful in predicting the responsiveness of locally advanced and metastatic disease to antihormonal therapies, with patients with receptor-positive disease (ER+) more frequently responding to treatment than when their tumours are receptor negative (ER−). Unfortunately, the association between ER expression and endocrine sensitivity is not absolute and a search for additional markers of oestrogen responsiveness has been pursued. The cellular products of oestrogen-regulated genes are ideal candidates for such markers since their detection in ER+ disease would suggest a functional ER system and hence a sensitivity to endocrine treatments.

In light of the above, we have cloned 10 oestrogen-induced sequences from the ZR-75-1 (pLIV1-pLIV2) and T-47D (pSYD1-8) human breast cancer cell lines [1, 2]. One of these, pLIV2, has been shown to be identical to the oestrogen-regulated pS2 gene [3]. These have been used to identify their corresponding mRNA in a range of oestrogen-responsive tissues, including human breast cancers. The current paper reports the expression of four of these genes; pLIV1, pS2 (pLIV2), pSYD3 and pSYD8 in 118 primary breast tumours which have been immunohistochemically assayed for ER, epidermal growth factor receptors (EGFR) and the cell proliferation protein detected using the Ki67 antibody. Each of these immunohistochemically defined endpoints has been previously shown by our group to relate to the sensitivity of breast tumours to endocrine therapy [4, 5].

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MATERIALS AND METHODS

Samples of primary tumour tissue were rapidly frozen upon excision, stored at −70°C and transported in dry ice to the Tenovus Cancer Centre for analysis. Representative portions of the tissue were blocked for cryostat sectioning and subsequent immunohistochemistry or reserved for RNA extraction and subsequent hybridisation analysis.

RNA extraction and electrophoresis

Tissue was homogenised in liquid nitrogen and resuspended in 4M guanidinium thiocynate containing 1% 2-mercaptoethanol. Total RNA was obtained by sedimentation through 5.7 mol/l caesium cloride and stored in RNase free water at −70°C.

Electrophoresis of RNA and northern transfer were performed as described previously [1].

Labelling of cDNA and hybridisation analysis

pLIV1, pS2 (pLIV2), pSYD3 and pSYD8 were labelled using [^{32}P]dCTP (300 Ci/mmol Amersham, U.K.) and hybridisation analysis performed as described previously [2]. Densitometric scans of X-ray film were performed using a Biorad 620 video densitometer. Cut-off values were assigned for all genes and were based on the need to account for both background hybridisation and the level of basal expression found in oestrogen-deprived human breast cancer cells [2]. The relative cut-off values for pLIV1, pS2, pSYD3 and pSYD8 were 1.0, 0.1, 1.75 and 1.75, respectively.

Immunostaining and assay controls

The immocytochemical assays (ICA) for ER [6], EGFR [5] and Ki67 [7] have been previously described. The assays were performed on adjacent sections that were cut and fixed simultaneously and carried out within 2 weeks of each other. Control antisera were included in the ER-ICA and EGFR-ICA to check for non-specific staining while in the Ki67 assay, substitution of primary antibody with phosphate-buffered saline was included as control. Sections previously found to be positive for each assay were included within each staining run to control for interassay variance.

Specimen evaluation and correlation with gene expression

All specimen evaluation was performed on an Olympus microscope (BH2) first using an occular magnification of × 10 in order to enable the localisation and subsequent avoidance of normal and benign areas within the section. This initial examination also allows the heterogeneity of immunostaining within the tumour components to be assessed and thus ensures that adequate sampling of all areas is performed. Subsequent evaluations were carried out using an ocular magnification of × 40 by two personnel using a dual viewing attachment to the microscope. Control slides were checked for non-specific binding before assessing the percentage of tumour cells stained by the primary antibody (minimum 2000 cells evaluated).

Tumours were classified as ER- and EGFR-positive if > 2% of tumour cells showed specific immunostaining. In order to facilitate a quantitative analysis of the ER and EGFR data, receptor positivity was further classified as receptor rich (ER++) or poor (ER+) based on the cellular positivity and the immunostaining density per cell. Tumours with greater than 70% of cells staining intensely for ER or 60% for EGFR were classified as receptor rich. Staining evaluation for the Ki67 antibody was similarly based on the proportion of tumour cells immunostaining with categories being defined as negative (< or equal to 10% cells staining), moderately positive (11–30% cells staining) and strongly positive (> 30% cells staining). The cut-off points for the EGFR [5], ER and Ki67 [4] assays have been previously shown by our group to relate to the endocrine responsiveness of breast cancer.

Statistical analysis

χ^2 contingency tables with Yates correction factor, where appropriate, and non-parametric Mann–Whitney U-tests were used to compare subgroups of the tumour population.

RESULTS

Northern analysis of tumour RNA with pLIV1, pS2, pSYD3 and pSYD8

Figure 1 shows a representative autoradiogram of RNA hybridised with radiolabelled pLIV1, pS2, pSYD3 and pSYD8 cDNAs as described in Materials and Methods. As previously demonstrated, pLIV1 recognises two major mRNA sequences at 4.4 and 2.3 kb and pS2 a 0.6 kb mRNA species [1], while both pSYD3 and pSYD8 hybridise to multiple mRNA [2]. For pSYD3 the major mRNA species is at 1.9 kb with less intense hybridisation observed at 1.7, 1.0 and 0.5 kb whereas the pSYD8 cDNA recognises two mRNA at 1.9 and 1.0 kb.

Associations between pLIV1, pS2, pSYD3 and pSYD8 expression and ER status

ER values were determined for all 118 breast tumours by immunohistochemical analysis.

In this study, 86 tumours were ER positive (73%) and 32 were ER negative (27%). pLIV1 mRNA was detectable in 46 (39%) of all tumours and was significantly associated with ER-positive disease (χ^2 = 13.69, P = 0.001), where 42 (49%) of ER-positive tumours were also pLIV1-positive compared to only 4 (12%) in ER-negative cancers (Fig. 2a). Similarly, pLIV1 mRNA levels were significantly elevated in ER-positive compared to ER-negative disease (Fig. 3, MW P = 0.0005).

Subdivision of the ER-positive tumours into those which showed moderate and high degrees of ER positivity failed to reveal any significant associations between ER concentration and either pLIV1 status or mRNA levels (data not illustrated).

pS2 mRNA was detected in 39 tumours and like pLIV1 was significantly associated with ER+ disease (Fig. 2b), where a total of 36 (42%) ER-positive tumours expressed pS2, compared to only 3 (9%) patients with ER-negative disease (χ^2 = 11.12, P = 0.001). pS2 mRNA levels were also significantly greater (MW P = 0.05) in ER-positive compared to ER-negative breast cancers (Fig. 3). Further subdivisions into moderately and

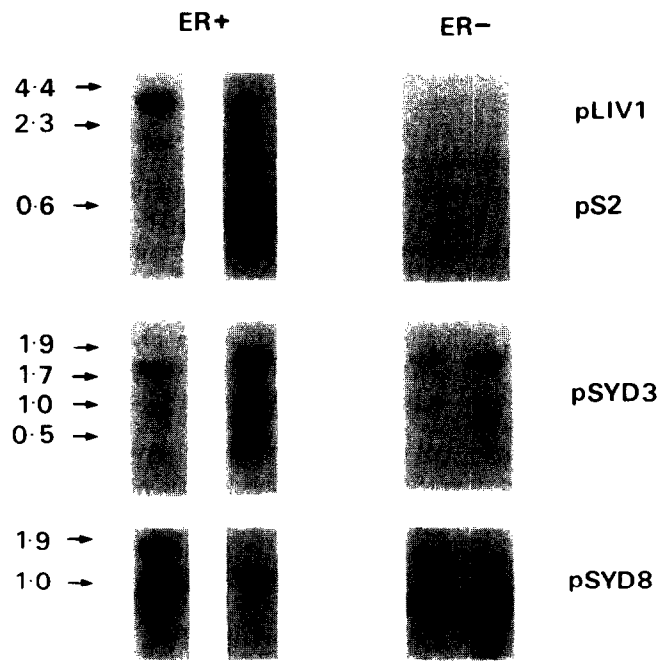

Fig. 1. A representative autoradiogram of tumour RNA following hybridisation analysis with pLIV1, pS2, pSYD3 and pSYD8.

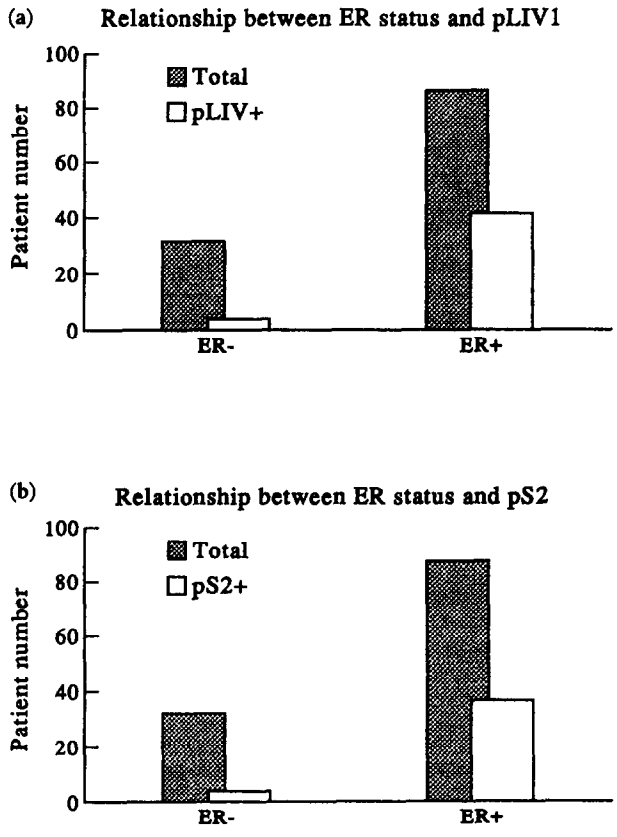

Fig. 2. The proportion of tumours which were ER positive (+) or negative (−), as shown by immunohistochemistry, and either pLIV1 (a) or pS2 (b) positive (+) or negative (−) as determined by hybridisation analysis.

highly ER-positive disease failed to identify an association between ER level and pS2 status or mRNA levels (data not illustrated).

When the levels of pLIV1 and pS2 were analysed together (Fig. 4), 25 (21%) breast cancers were pLIV1- and pS2-positive whereas 58 (48%) tumours were negative for both pLIV1 and pS2. The remaining 35 (31%) breast cancers showed mixed pLIVI/pS2 phenotypes, with a total of 21 (19%) being positive for pLIV1 and 14 (12%) being positive for pS2+. Subdivision of these groups according to ER status showed a highly significant trend for the expression of pS2 and pLIV1 and ER positivity ($\chi^2 = 18.5$ $P = 0.001$) with the ER+pLIV1+pS2+ phenotype being observed in 29% of ER+ tumours. In ER-positive tumours the mRNA levels of pLIV1 and pS2 were significantly elevated when expressed together (i.e. in the pLIV1+/pS2+ phenotype) in comparison with the levels found in the pLIV1+pS2− ($P = 0.03$) or pLIV1−pS2+ ($P = 0.01$) mixed phenotypes. Co-expression of pLIV1 and pS2 was not observed in any of the 32 ER-negative cancers.

In contrast to pLIV1 and pS2, pSYD3 and pSYD8 mRNA was detected in almost all of the tumours and did not show any relationship with ER status (Fig. 3) or level (data not shown).

Quantification of EGFR protein and correlation to ER, pLIV1, pS2, pSYD3 and pSYD8 expression

A highly significant inverse association ($\chi^2 = 67.5$, $P = >0.0001$) between ER and EGFR status was observed in the 118 tumours examined (Fig. 5). This relationship was also observed for both pLIV1 and pS2 (Fig. 6), with their mRNA being detected predominently in EGFR-negative disease ($\chi^2 = 6.64$, $P = 0.01$ and $\chi^2 = 3.34$, $P = 0.08$, respectively). Quantification of the pLIV1 and pS2 mRNA levels revealed significantly higher hybridisation values (MW $P = 0.01$ and $P = 0.05$, respectively, in EGFR-negative tumours compared to the levels observed in EGFR-positive disease). Subdivision of the EGFR-positive tumours into those showing moderate or high levels of immunostaining did not reveal any associations between breast tumour EGFR levels and pLIV1 or pS2 positivity or mRNA levels (data not illustrated). In addition, a significant trend was observed for the absence of co-expressed pLIV1 and pS2 in EGFR+ disease ($\chi^2 = 8.027$, $P = 0.05$).

Analysis of the data in respect to both ER and EGFR status revealed that only 10% of tumours deviated from the reciprocal relationship between ER and EGFR. These tumours were either ER- and EGFR-positive (2.5%) or negative (7.5%). While pLIV1 and pS2 were detactable in two of the three ER- and EGFR-positive cancers (with co-expression observed in one of these), both mRNAs were undetectable in ER- and EGFR-negative cells.

Although no significant associations were observed between pSYD3 and pSYD8 positivity and EGFR expression, pSYD8 was found at lower levels ($\chi^2 = 3.68$, $P = 0.06$) in moderately EGFR-positive disease (Fig. 6).

Correlation of pLIV1, pS2, pSYD3 and pSYD8 with Ki67

The expression of all four genes in relation to Ki67 was determined (Fig. 7). Three genes: pLIV1, pS2 and pSYD8, failed to reveal any significant relationship with Ki67 staining. The incidence of pSYD3 positivity and mRNA levels were greater in Ki67-positive tissue compared to Ki67-negative disease ($\chi^2 = 7.245$, $P = 0.01$ and MW $P = 0.001$, respectively). Subdivision of Ki67 levels into moderately proliferating and highly proliferating tumour types did not show any further significant trends for pSYD3 expression (data not illustrated). Analysis of the Ki67 data in relation to ER status did not reveal any further significant observations.

DISCUSSION

We have measured the expression of four oestrogen responsive genes in 118 immunohistochemically defined primary breast tumours by northern analysis. While all four genes are induced by physiological levels of oestrogen in ER-positive human breast cancer cell lines [1, 2], they are differentially expressed in clinical breast tumour samples. Two patterns of gene expression are discernible, however.

The first is represented by pLIV1 and pS2 (pLIV2) which are heterogeneously expressed in the breast tumour population and show a significant association with ER status. While no further increase in either pLIV1 or pS2 expression was observed with increasing ER positivity, presumably due to either unoccupied receptor or promoter-specific differences, previous studies from a number of laboratories [8], including our own [9], have demonstrated that a patient's likelihood to respond to endocrine therapy is closely associated with ER+ disease. Consequently, the detection of pLIV1 and pS2 in ER+ tumours suggests a functional receptor system and hence a sensitivity to endocrine intervention. This relationship has been previously established for the progesterone receptor which is also inducible in breast cancer cells [10] and when detected in ER+ clinical samples indicates an 80% probability of response to endocrine therapy compared to 10% in ER- and PR-negative tumours. The lack of an association between increasing ER levels and pLIV1 and pS2

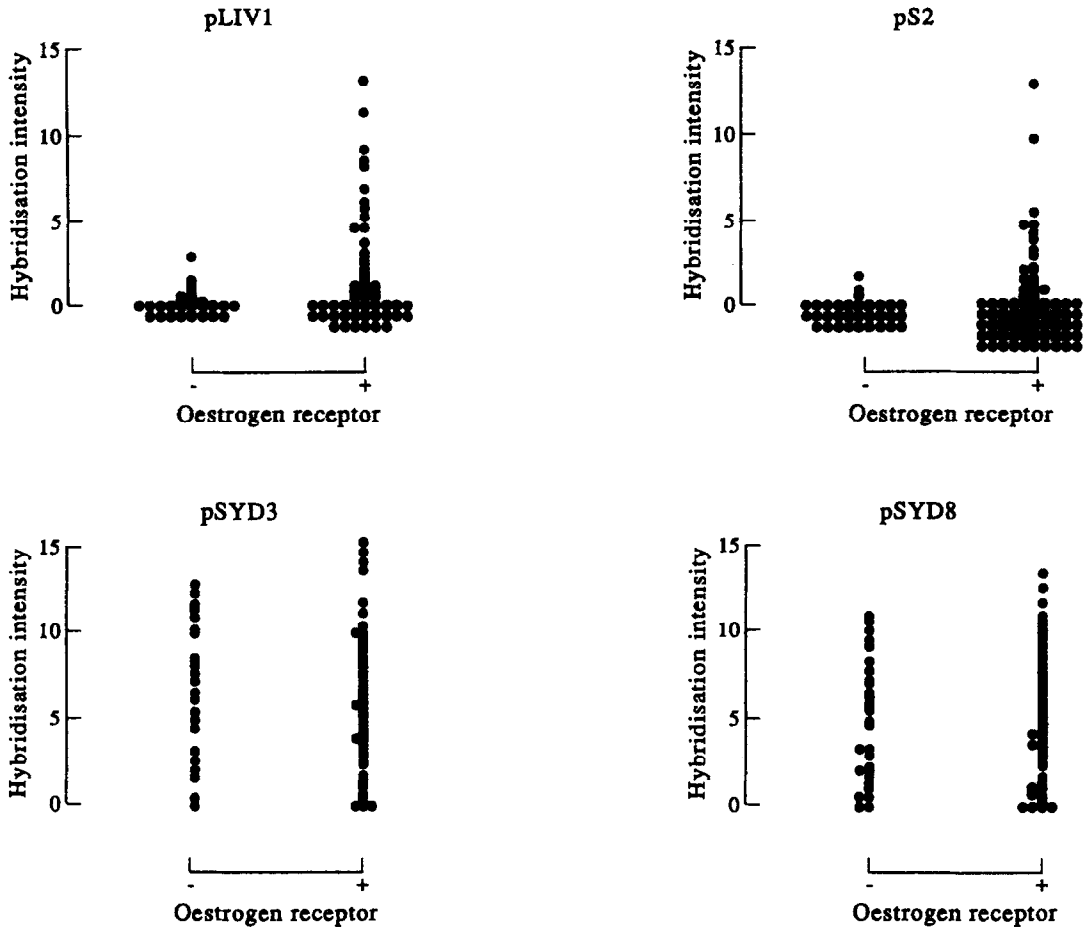

Fig. 3. mRNA levels (as shown by hybridisation intensity) were determined by northern analysis for pLIV1, pS2, pSYD3 and pSYD8 and plotted against ER status.

expression suggests that transcription is unaffected by receptor concentration.

Interestingly, in this study, pLIV1 and pS2 were not always co-expressed in ER+ disease with all four possible subgroups identified, ER+pLIV1−pS2−, ER+pLIV1−pS2+, ER+pLIV1+pS2− and ER+pLIV1+pS2+. A number of similar subgroups have been found in ER+ disease for both the pS2 and PR genes [11] and it may be that explanations for the observed subgroups lie in two mechanistically divergent areas which are linked through the promoter strengths of individual oestrogen-regulated genes. It is well established, for example, that the promoters of oestrogen-regulated genes differ from one another.

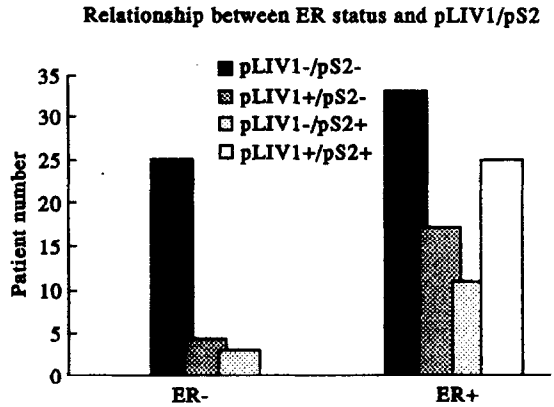

Fig. 4. The levels of both pLIV1 and pS2 were analysed together and expressed in relation to ER status. Four tumour subgroups were identified (pLIV1+pS2+, pLIV1+pS2−, pLIV1−pS2+ and pLIV1−pS2−) and the number of tumours in each subgroup for either ER+ or ER− disease were plotted.

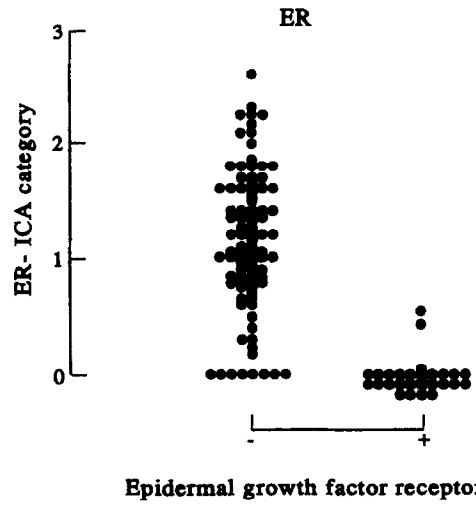

Fig. 5. The relationship between ER immunostaining (by ER category, as decribed in Materials and Methods) and EGFR status (positivity + or negativity −).

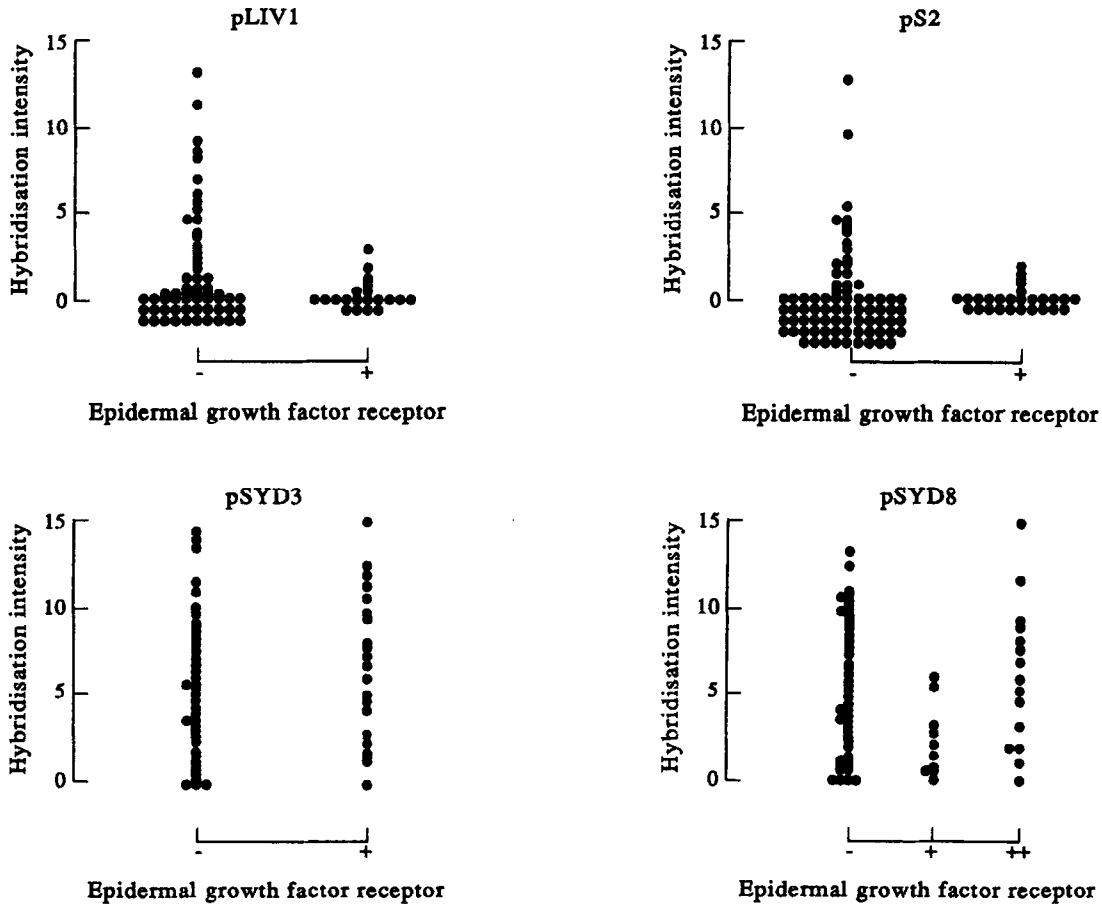

Fig. 6. mRNA levels (as shown by hybridisation intensity) for pLIV1, pS2, pSYD3 and pSYD8 were determined for all 118 tumours by northern analysis and plotted against the EGFR status of the tumour. For pSYD8, further subdivision of the tumour EGFR status is shown with mRNA levels recorded for EGFR negative (−), moderately positive (+) and strongly positive (++) tumours as described in Materials and Methods.

Both May and Westley [12] and Manning *et al.* [2, 13] have demonstrated variations in the magnitude of oestrogen inducibility for a series of responsive genes. Furthermore, the response of oestrogen response elements (ERE) to tamoxifen varies between different oestrogen inducible genes [14]. Consequently, any alterations in either the availability of oestrogens or ER integrity could effect transcription from either pLIV1, pS2 or the PR gene. Both mechanisms would be dependent on the ability of the promoters to differentiate efficiently between high and low levels of activated ER or to discriminate between mutated and wild type ER forms of the receptor.

The clinical consequences of these two mechanisms are diverse, since if the former predominates and pLIV1 and pS2 are low in our study because of inadequate oestrogenic stimulation then a functional ER system could still exist and tumours may still retain endocrine sensitivity. Alternatively, if detrimental alterations have occurred in the ER, then the reduction or complete loss of expression of previously oestrogen-regulated genes may be symptomatic of a loss of responsiveness to endocrine measures.

Both these clinical scenarios have been shown to exist following ER and PR measurements where the pooling of data from nine centres has indicated that approximately half of ER+ tumours were PR negative [15]. Significantly, the absence of the PR in these tumours did not totally preclude a response to endocrine therapies, with 34% of such patients deriving some measurable benefit [16].

Unfortunately, our results showing significantly lower pLIV1 or pS2 mRNA levels in the ER+pLIV1−pS2+ and ER+pLIV1+pS2− mixed phenotypes compared to the levels observed when pLIV1 and pS2 are co-expressed cannot, in themselves, differentiate between the mechanisms. However, cell culture studies performed on the ER+ MCF-7 cell line have shown that following oestrogen withdrawal pLIV1 mRNA, but not pS2, remains detectable, i.e. the pLIV1-positive pS2-negative phenotype predominates under these conditions. In order to differentiate between these two possibilities pLIV1 and pS2 mRNA levels in tumour cells prior and during antiestrogen therapy could be determined or alternatively, genes which are negatively regulated by oestrogen, e.g. pMGT1 [29], could be measured.

Our results have also demonstrated that while the majority of ER-negative tumours were pLIV1- and pS2-negative (and may, therefore, be considered hormone insensitive), a small number had low but detectable levels of either pLIV1 or pS2 (co-expression of pLIV1 and pS2 was not detected in ER-negative disease), possibly suggesting a false-negative ER determination. Indeed, recent studies have identified the ER as a cell cycle-related protein which is not readily detected in tumours or normal breast tissue with low growth fractions [17]. Since these tissues are invariably hormone sensitive [4], the detection of oestrogen regulated gene products may provide a means of further identifying this phenotype and differentiating them from truly autonomous cancers where patients have a very

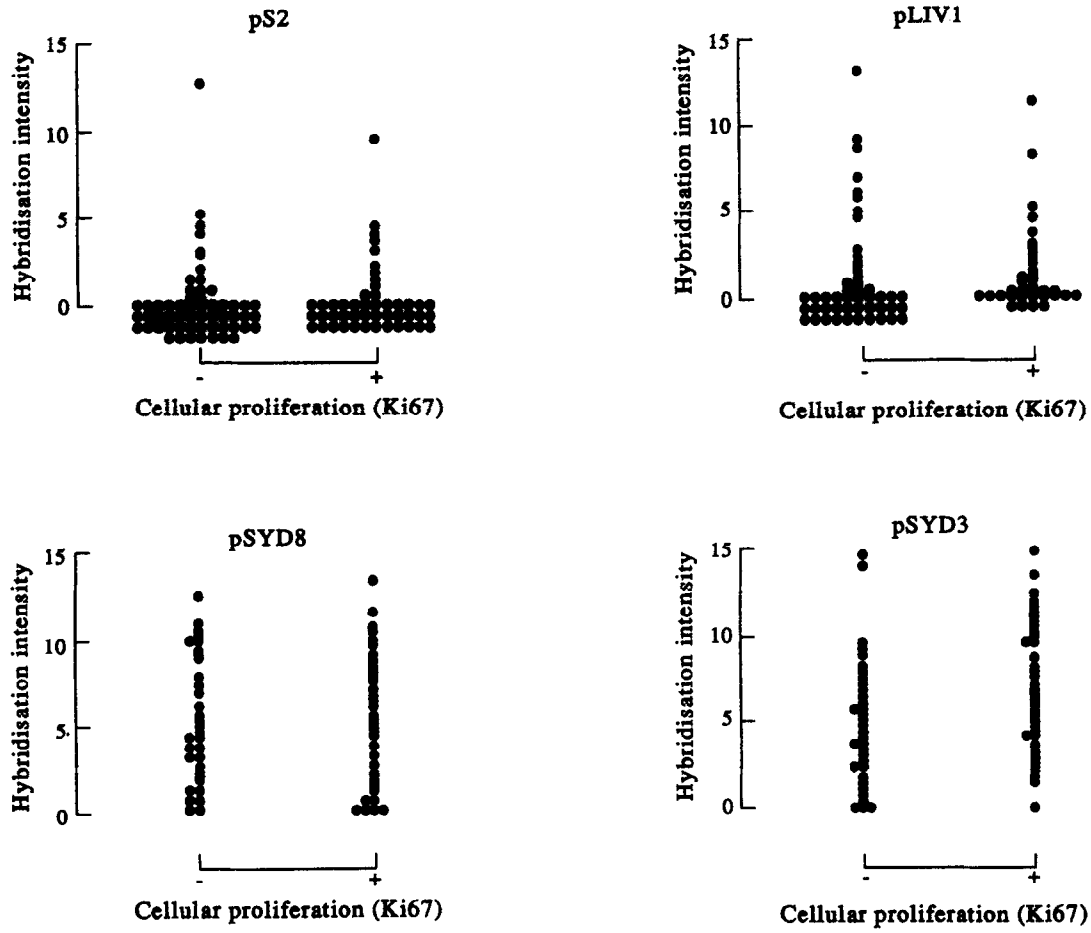

Fig. 7. mRNA levels (as shown by hybridisation intensity) for pLIV1, pS2, pSYD3 and pSYD8 were determined for all 118 tumours by northern analysis (as described in Materials and Methods) and plotted against Ki67 status.

unfavourable outlook [4, 5]. Alternatively, ER-tumours which express either pLIV1 or pS2, may in fact be receptor positive but in a form which is undetectable by the antibody used in the immunohistochemical assay. Fuqua *et al.* [18] have recently identified variant oestrogen receptors in tumours regarded as ER-negative but were PR-positive. Such receptors were unable to bind oestrogen but could constitutively activate transcription. Consequently, ER variants may exist within our tumour population that are undetectable in our assay and as such are classified as ER-negative but are sufficiently active to drive transcription from the pLIV1 or pS2 promoters.

Our observation demonstrating an inverse relationship between ER and EGFR parallels previous studies [19]. Indeed, this study has also revealed a similar inverse correlation between the expression of pLIV1 or pS2 and the EGFR. Further analysis of this observation demonstrated the complete absence of pLIV1 or pS2 mRNA in cells negative for both ER and EGFR (but not in ER+EGFR+tumours). Since ER−EGFR− tumours are frequently hormone sensitive [20] and are reminiscent of the situation observed in normal breast [21], it may be that pLIV1 and pS2 negativity, in certain circumstances, represent a favourable prognosis for the patient.

The second group of genes examined in the current study comprising pSYD3 and pSYD8 are expressed in the majority of primary breast cancers and show no detectable relationship to the ER. Although this result is somewhat surprising, since both genes are oestrogen inducible in human breast cancer cell lines where they are downregulated by antioestrognes, their behaviour is similar to that reported for cathepsin-D [22]. It is possible that explanations for these apparently anomalous observations again reside in the promoter regions of these genes with variations in the strength of the oestrogen inducible component being overridden by their responsiveness to other rgulatory elements. In this light, it is known that oestrogens stimulate the synthesis of a number of growth factors in breast cancer cell lines including one of the ligands for the EGFR, namely TGF-α [23]. Constitutive production of growth factors like TGF-α could potentially override a cells requirement for oestrogen leading to oestrogen-independent growth. This view is consistent with the observations showing that cathepsin-D is inducible, in oestrogen responsive cells, by EGF and insulin-like growth factors [24, 25] and that this may be reproduced for pSYD3 (unpublished observations). Indeed, since pSYD3 levels are frequently elevated in rapidly proliferating tumours and over-expressed in others (data not included), its expression in ER+ disease may provide an additional marker for the loss of hormone sensitivity.

While growth factors can substitute for some of the growth promoting properties of oestrogen in ER+ cell lines, it is apparent that this mechanism is insufficient to explain the loss of oestrogen-dependent growth. For example, antibodies raised against TGF and its receptor do not inhibit oestrogen-induced proliferation [26]. However, the presence of EGF mRNA in 83% of breast cancers [27] and our own observations demonstrating TGF-α in approximately 80% of the tumours used in this study

[28] suggests a significant role in cell proliferation and a potential mechanism by which pSYD3 and 8 are expressed in the majority of tumours.

From these initial studies monitoring a panel of oestrogen-responsive genes isolated from breast cancer cells, it is apparent that their expression in clinical specimens is complex and, in themselves, cannot fully describe the endocrine responsiveness of breast cancer. They do, however, provide a useful mechanism of investigating the control of oestrogen-regulated events in normal and neoplastic cells, such that when used in conjunction with other parameters, which relate to the endocrine sensitivity of breast cancers, may provide clinically useful information. As such, our current studies are directed towards sequencing these genes (and their promoter regions) investigating their regulation by hormones and growth factors and exploring their prognostic significance and relationships to a broader spectrum of factors which may govern the sensitivity of breast cancer cells to endocrine treatments.

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