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Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene *ERBB2*

(immunocytochemical staining of sections/estrogen-dependent breast cancer diagnosis/tumor markers/human uterus/mRNA blotting)

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ABSTRACT **The expression of the pS2 gene, which is induced by estrogen in the breast cancer cell line MCF-7, has been investigated in breast cancers by using pS2 mRNA determination in tumor specimens and immunocytochemistry to identify pS2 protein in paraffin-embedded sections. Using these assays we show that determination of pS2 gene expression allows the definition of subclasses of estrogen-receptor-containing breast cancers that may be used to more precisely identify estrogen-dependent tumors. Tumor specimens have also been analyzed for the presence of mRNAs for the estrogen receptor and for the *ERBB2* oncogene. No evidence for the presence of truncated forms of estrogen-receptor mRNA has been found, and overexpression of the *ERBB2* oncogene did not correlate with the steroid receptor status or pS2 gene expression.**

The hormone dependence of some human breast cancers is well recognized. Most, but not all, patients with estrogen-receptor (ER)-containing tumors receive objective benefit from hormonal therapy (1). The failure of 30–40% of the patients with ER-rich cancers to respond to endocrine therapy was ascribed to either tumor heterogeneity or to the fact that the ERs might be nonfunctional (1). Horwitz and McGuire (2) suggested that determining the progesterone-receptor (PR) content of tumors might allow the identification of tumors with functional receptors, because PR expression is known to be estrogen-dependent in normal reproductive tissue and in the breast cancer cell line MCF-7 (refs. 2 and 3 and references therein). In fact, not all (≈80%) of ER-positive and PR-positive breast cancers are hormonally responsive (1), indicating a need for additional markers of hormone responsiveness to identify more readily those breast cancers that can benefit from hormonal therapy.

The expression of the human pS2 gene is specifically controlled by estrogens at the transcriptional level in the cancer cell line MCF-7 (4, 5). The pS2 protein is a small secreted protein of unknown function (6, 7). The availability of a cDNA probe for pS2 mRNA (4) and of specific polyclonal antibodies against the pS2 protein (7) has prompted us to investigate whether pS2 gene expression could be an additional marker for estrogen-dependent breast cancers. In addition, we have analyzed tumor specimens for the possible presence of "mutated" ER mRNA (8, 9) and overexpression of the *ERBB2* oncogene (*HER2*/neu, a member of the *ERBB*-like oncogene family) whose amplification has been reported in a subclass of breast cancers (10, 11).

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MATERIALS AND METHODS

Breast Cancer and Other Tissues. The surgical specimen was cut in sections as described (12). For each section taken for biochemical receptor determination or RNA analysis, adjoining sections were taken for histological examination, immunocytochemical detection of ER, PR, and pS2 protein, and determination of the tumor cellularity index (12). Samples for biochemical determination of ER, PR, and RNA analysis were immediately frozen in liquid nitrogen, whereas samples for ER immunocytochemical assay (ICA) and PR ICA were frozen in 2-methylbutane at −180°C, and those for pS2 ICA were fixed in 10% buffered formalin (Sigma) for 24 hr. All other tissues or cells were immediately frozen in liquid nitrogen.

pS2 Protein Immunocytochemical Staining in Paraffin Sections and Other Immunocytochemical Assays. Formalin-fixed samples were embedded in paraffin, sections 5 μm thick were cut, deparaffinized with toluene, and rinsed thoroughly with absolute ethanol. After washing with water and isotonic phosphate-buffered saline, pH 7.4 (PBS), the sections were incubated with sheep serum [2.5% (vol/vol) in PBS containing 0.5% bovine gamma globulin] to reduce the nonspecific staining. After incubation with the anti-pS2 peptide rabbit polyclonal antibodies (1:640 dilution), the sections were incubated successively with sheep anti-rabbit IgG (1:10 dilution; Ortho Diagnostics), and rabbit peroxidase-antiperoxidase (1:50 dilution; Ortho Diagnostic). Each incubation was followed by washing with PBS. After a final rinse in PBS, the sections were incubated with 3,3′-diaminobenzidine and counterstained with hematoxylin (1:10 dilution; Merck). ER ICA and PR ICA were performed on frozen sections using the Abbott and Transbio (Paris, France) monoclonal antibody kits, respectively, according to the procedure recommended by the suppliers. Staining intensity was estimated on a 4-point scale (0–3+; shown in parentheses following the protein), and the proportion of positively stained tumor cells was determined as a percentage of total tumor cells (1–100%). The staining index was obtained by multiplying the percentage of stained cells by the staining score (1–300).

RNA Analysis. RNA purification directly from frozen sections, RNA transfer to diazobenzyloxymethyl-paper (DBM-paper) and hybridization with ^{32}P-labeled nick-translated cDNA probes (≈10^8 cpm/μg of DNA) were performed as described (4, 6). pS2 mRNA, ER mRNA, and ERBB2 mRNA were estimated semiquantitatively taking into account the hybridization with the control 36B4 cDNA probe

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; ICA, immunocytochemical assay; DCC, dextran-coated charcoal steroid-binding assay; DBM-paper, diazobenzyloxymethyl-paper.

(which reflects the amount of RNA actually loaded on the gel and to some extent the cellularity of the tissue specimen, ref. 4) and the intensity of hybridization with RNA prepared from MCF-7 cells that express pS2, 36B4, and ER genes but poorly express the *ERBB2* oncogene (14). Hybridization scores were allocated on a 5-point scale (not detectable, just detectable, low, moderate, and high intensities: 0, ±, 1+, 2+, and 3+, respectively; shown in parentheses following the mRNA).

Other Materials and Methods. The raising of a rabbit polyclonal antiserum against pS2 synthetic peptide has been described (7). ER and PR dextran-coated charcoal steroid-binding assay (DCC) were carried out as described (12).

RESULTS AND DISCUSSION

pS2 mRNA Is Present Only in a Subset of Breast Cancers: Comparison with ER and ERBB2 mRNAs. The presence of pS2 mRNA was investigated by RNA blotting of individual RNA preparations purified from >300 tumor specimens. DBM-blots were simultaneously hybridized with a cDNA probe for the ubiquitous 36B4 mRNA. Some representative cases are shown in Figs. 1 and 2. pS2 mRNA [0.6 kilobases (kb)] was present in a number of breast cancers at various levels (at high levels in Fig. 1, lane 8, and Fig. 2, lane 4; at low levels in Fig. 2, lane 2), but in some cases was not detectable (e.g., Fig. 1, lane 4, and Fig. 2, lanes 1 and 3). pS2 mRNA was also expressed at various levels in some metastatic axillary nodes of pS2 mRNA-positive primary tumors (e.g., Fig. 1, lane 7) but was never observed in metastatic nodes of pS2 mRNA-negative cancers (data not shown). On the other hand, pS2 mRNA was not detected in benign breast tumors (e.g., fibroadenomas; see Fig. 1, lanes 5 and 6) or in normal axillary nodes (e.g., Fig. 1, lane 3, and Fig. 2, lane 5). pS2 mRNA was not detected in any of a variety of other normal or tumor tissues and cells, regardless of the presence of ER.

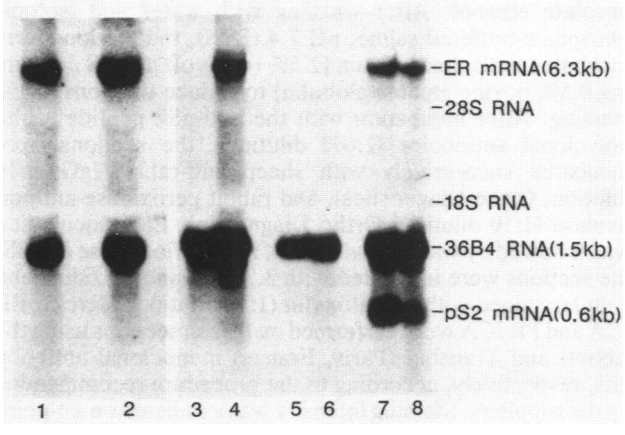

FIG. 1. RNA blot analysis of pS2 and ER mRNAs in various normal tissues and tumors. RNA was prepared, electrophoresed, blotted on DBM-paper, and hybridized with pS2 and 36B4 cDNA probes (4, 13). After autoradiography (24 hr), the blot was dehybridized and hybridized with an ER cDNA probe (refs. 8 and 9; λOR8 clone) before a second autoradiography (3 days). The figure shows the superimposition of the two autoradiograms. The source of the RNA (≈20 μg) was as follows. Lanes: 1, normal endometrium; 2, endometrial carcinoma; 3, normal axillary node of patient A; 4, breast carcinoma [patient A, ductal epithelioma grade II with metastatic axillary nodes, ER DCC of 46 fmol/mg, ER ICA(3+), PR DCC(0), PR ICA(0), and pS2 ICA(0)]; 5 and 6, two ER ICA(0) and pS2 ICA(0) breast fibroadenomas; 7, metastatic axillary node of patient B [ER DCC, 40 fmol/mg, PR DCC 1370 fmol/mg, ER ICA(3+), PR ICA(3+), and pS2 ICA(3+)]; 8, breast carcinoma [patient B, ductal epithelioma grade II with ER DCC, 33 fmol/mg; PR DCC, 1630 fmol/mg; ER ICA(3+); PR ICA(3+); and pS2 ICA(3+)].

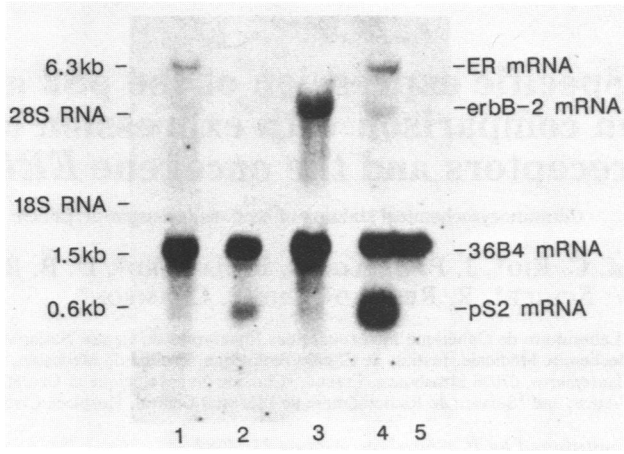

FIG. 2. RNA blot analysis of pS2, ER, and ERBB2 mRNAs in several breast carcinomas and in a normal axillary lymph node. RNA was prepared, electrophoresed, blotted on DBM-paper, and hybridized with pS2, 36B4, and ER cDNA probes as described in Fig. 1, except that after a second dehybridization the filter was hybridized with an ERBB2 cDNA probe (internal 1.6-kb *Eco*RI fragment in ref. 14) before a third autoradiography (3 days). The figure corresponds to the superimposition of the three autoradiograms. The source of the RNA (≈20 μg) was as follows. Lanes: 1, breast carcinoma [patient C, ductal epithelioma grade II with no axillary node metastasis (0/9), ER DCC(0), ER ICA(1+), PR DCC(0), and PR ICA(0), and pS2 ICA(0)]; 2, breast carcinoma [patient D, ductal epithelioma grade II with no axillary node metastasis (0/10), only 3% tumor cellularity, ER DCC(0), ER ICA(3+), PR DCC(0), PR ICA(0), and pS2 ICA(3+) in the tumor cells]; 3, breast carcinoma [patient E, ductal epithelioma grade II with axillary lymph node metastasis (1/5), ER DCC(0), ER ICA(0), PR DCC(0), PR ICA(0), and pS2 ICA(0)]; 4, breast carcinoma [patient F, ductal epithelioma grade I with no axillary node metastasis (0/10) and ER DCC of 12 fmol/mg, ER ICA(1+), PR DCC of 334 fmol/mg, PR ICA not done, pS2 ICA(3+)]; 5, normal axillary node of patient F (ER-, PR-, and pS2-negative using all types of assays).

For instance, it could not be detected in endometrium or in carcinomas of the endometrium (Fig. 1, lanes 1 and 2). Thus the presence of pS2 mRNA appears to be highly specific to a subclass of primary breast cancers and axillary node metastases.

The presence of ER mRNA was detected by hybridization with an ER cDNA probe using the same DBM-blots. Some specimens from breast cancers and metastatic nodes contained relatively high amounts of ER mRNA (Fig. 1, lanes 4, 7, and 8), whereas others had low (Fig. 2, lanes 1 and 4) or not detectable (Fig. 2, lanes 2 and 3) levels of ER mRNA. In general there was a good correlation (data not shown) between the ER ICA staining index, the ER mRNA hybridization score, and the biochemical determination using ER DCC. ER mRNA was usually not detected under the present assay conditions when the ER DCC was lower than 20 fmol/mg of protein or when the ER-ICA staining index was <30. Such a case is presented in Fig. 2, lane 2, where only 3% of the cells were tumor cells with an ER-ICA staining score of 3+. Note that ER mRNA was detected in some specimens (e.g., Fig. 2, lane 1) that were ER DCC-negative, but ER ICA-positive, highlighting limitations of the ER-DCC determination.

In all of the tumors analyzed (114 tumors), a single ER mRNA band ≈6.3 kb long, as expected from our determinations (8, 9), was seen in both cancer specimens and metastatic nodes. ER mRNA present in normal and tumoral endometrium had a similar length (Fig. 1). Thus, ER mRNA is apparently not grossly altered in the ER-positive breast cancers. In this respect, we note that we have not found a

single case where ER mRNA was present, but the ER protein was absent when assayed by ER ICA.

ERBB2 mRNA was also assayed on the same DMB-blots. The 5-kb ERBB2 mRNA (14) was overexpressed at various levels in ≈20% of all breast cancers [i.e., at levels higher than in MCF-7 cells where it is considered not to be overexpressed (14)]. Representative cases of "high" and "just detectable" overexpression are shown in Fig. 2, lanes 3 and 4. However,

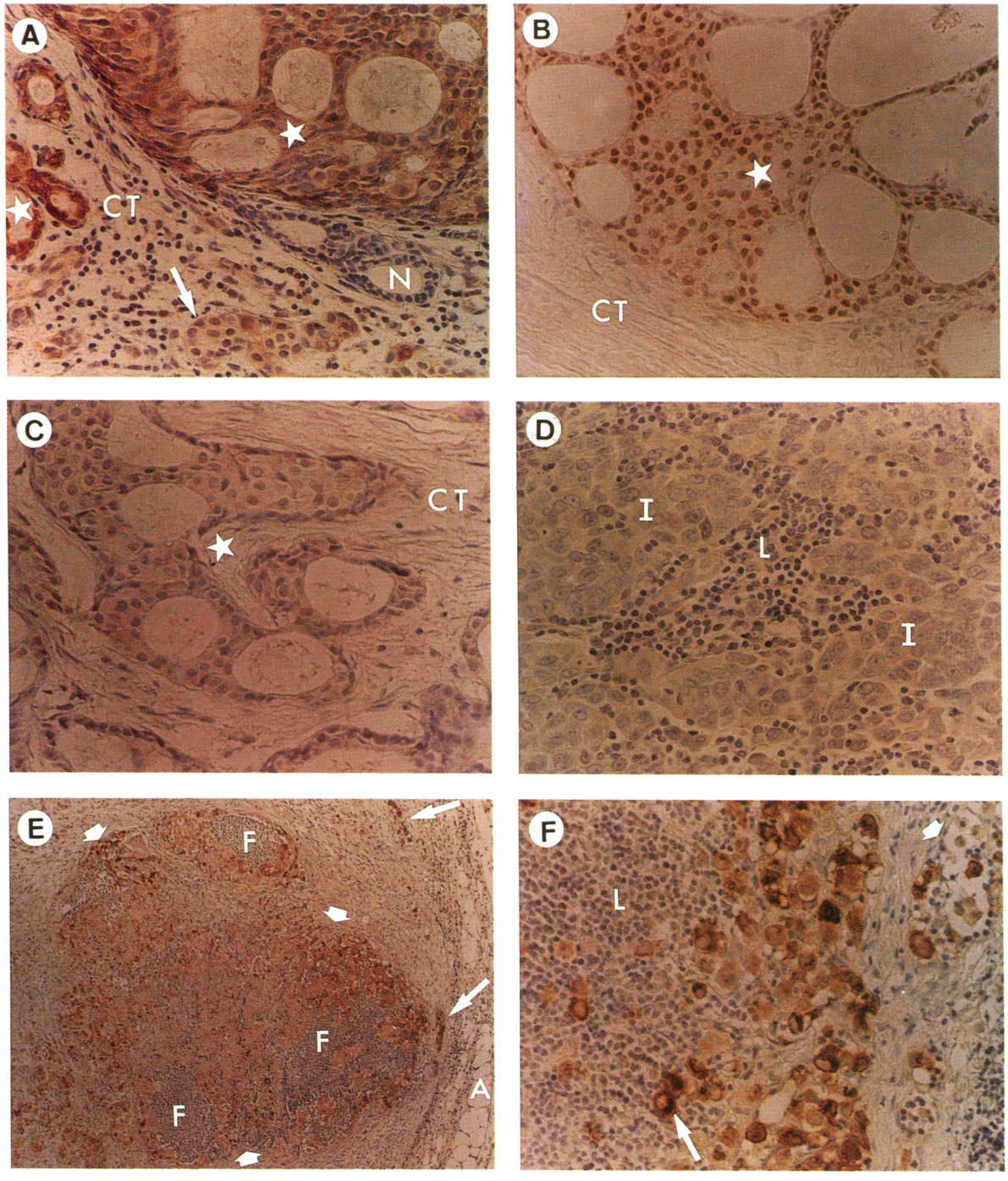

FIG. 3. Indirect immunoperoxidase staining of the pS2 protein in paraffin sections and of the ER in frozen sections. (*A*) pS2-positive tumor cells of a ductal carcinoma; the specific pS2 protein staining, which shows various intensities, is located in the cytoplasm of the tumor cells and the normal ductulary epithelium is not stained (N). Symbols are as follows: stars, intraepithelial component of the tumor exhibiting a cribriform pattern; arrow, infiltrative component in a trabecular pattern within the connective tissue (CT). (*B*) ER-positive tumor cells in frozen sections of the same ductal carcinoma. Symbols are as in *A*; the staining is nuclear and exhibits various intensities. (*C*) Almost complete disappearance of the pS2 protein staining when a section similar to that shown in *A* was stained in the presence of the pS2 peptide at 15 ng/ml that was used to raise the antibodies. (*D*) Global lack of pS2 protein staining in a paraffin section of a poorly differentiated invasive ductal carcinoma (I) that was negative for pS2 gene expression. L, lymph cell infiltrate. (*E*) pS2-positive cells in a section of a metastatic axillary node of a ductal carcinoma. Positive cells are scattered into a marginal sinus (arrow) and invade largely (arrowhead) between the follicles (F). Peripheric adipose tissue (A). (*F*) Same metastatic node at a higher magnification; note the variations in pS2 protein cytoplasmic staining, often with a perinuclear accentuation (arrow); the arrowhead points to pS2-positive tumor cells into a lymph channel. There are small lymphocytic cells (L) within an invaded follicle. The sections were counterstained with hematoxylin. (*A–D* and *F*, ×310; *E*, ×80.)

in contrast to pS2 expression, *ERBB2* overexpression in metastatic nodes was not necessarily linked to overexpression in the primary tumor and vice versa.

Immunocytochemical Detection of the pS2 Protein in Paraffin-Embedded Sections. The availability of a rabbit polyclonal antibody to the pS2 protein (7) prompted us to develop an ICA for the pS2 protein in tissue sections. Initial attempts with frozen sections gave mediocre results. However, the use of formalin-fixed paraffin-embedded sections resulted in high sensitivity, low background, and good preservation of the histopathological structure (Fig. 3). In contrast to the specific ER ICA immunoperoxidase staining that appeared to be exclusively nuclear (Fig. 3*B*), in agreement with reports (15, 16), the pS2 protein staining was cytoplasmic in breast cancer and metastatic node sections (Fig. 3 *A*, *E*, and *F*). However, the cytoplasmic distribution of the staining was uneven with often a perinuclear condensation (arrow in Fig. 3*F*) that may correspond to the Golgi apparatus, since the pS2 protein is known to be secreted (7). In addition, the intensity of staining was variable from one cell to another, even within a given area of a section (Fig. 3 *A* and *F*). The same was also found for the ER-specific nuclear staining (Fig. 3*B*). However, not all breast carcinomas were positive for the specific pS2 protein staining (Fig. 3*D*). The staining was specific for the pS2 protein as demonstrated by its suppression when competed with the synthetic peptide used to raise the antibodies (Fig. 3*C*). Furthermore, cells of normal ductulary epithelium ("N" in Fig. 3*A*) and benign breast tumors (data not shown) were not stained.

We observed a good correlation (data not shown) between the staining index for the pS2 protein and the intensity score for the pS2 mRNA as determined from RNA blot analysis, indicating that the presence of the pS2 protein most probably reflects increased transcription, as demonstrated using the breast cancer cell line MCF-7 (4, 5). That this induction is ER-dependent is strongly supported by the good correlation that exists between the staining index for the pS2 protein (as determined from pS2 ICA) and the staining index for the ER (as determined from ER ICA) (Figs. 4 and 5). Thus we conclude that the expression of the pS2 gene in breast cancers is most probably estrogen-dependent, as is the case in the MCF-7 cell line (4, 5, 7).

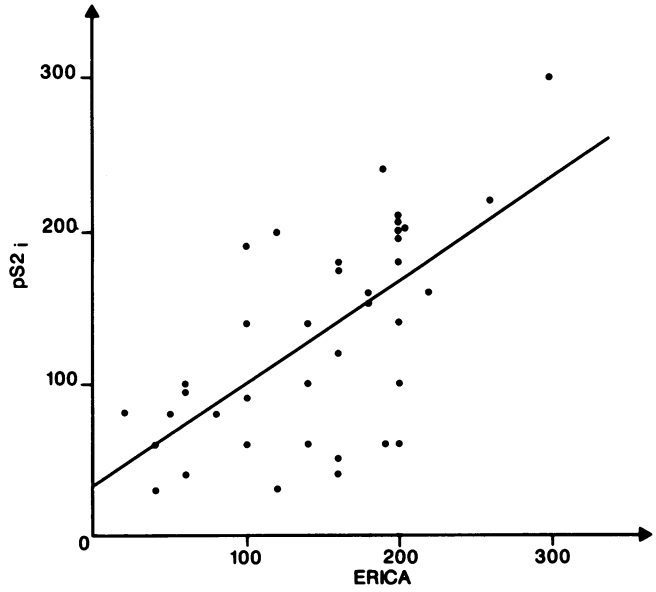

FIG. 4. pS2 protein-positive breast tumors: correlation between pS2 protein staining index ($pS2_i$) using pS2 ICA and ER staining index using ER ICA. Coefficient of regression = 0.65; $P < 0.01$; $n = 39$.

Comparative Expression of the pS2 Gene with that of the ER, PR, and *ERBB2*. Fig. 5 shows a summary of the results obtained for 180 breast cancer specimens for which all parameters have been unequivocally determined, with few exceptions. ER-positive and PR-positive specimens were positive using at least one of the ER and PR assays, pS2-positive specimens were positive for pS2 mRNA or pS2 ICA (usually for both), and *ERBB2*-overexpression was determined by mRNA analysis. For the sake of simplicity, no attempt was made to categorize these breast cancers taking into account staining index or mRNA intensity scores.

All breast cancers can be categorized in seven subclasses of unequal importance (Fig. 5). Seventy-two percent of the tumors were ER-positive, of which 88% were PR-positive and 12% were PR-negative. Interestingly, not all of the PR-positive cancers were pS2-positive, nor were all of the PR-negative cancers pS2-negative. In fact, only 62% of the ER-positive tumors were also positive for PR and pS2 expression [MCF-7 cell-like in this respect (13)], whereas 26% of the ER-positive tumors were PR-positive and pS2-negative [T-47D cell-like (5, 13)]. It is also noteworthy that some ER-positive, PR-negative tumors were either pS2-positive or pS2-negative. These observations are particularly interesting, since the transcription of both the PR and pS2 gene can be induced by estrogen in MCF-7 cells (ref. 5 and unpublished results). Thus, the simultaneous determination of ER, PR, and pS2 gene expression reveals an additional functional heterogeneity in ER-positive breast cancers. This may reflect either an heterogeneity in the ER itself, such that it can induce both the PR and pS2 genes, only the PR gene, only the pS2 gene, or none of them, or an heterogeneity in the estrogen-responsiveness of the PR and pS2 genes. On the other hand, ER-negative cancers appear strikingly homogenous with respect to PR and pS2 expression, since 96% of them did not express either of these two estrogen-inducible genes. *ERBB2* overexpression was equally distributed between the various subclasses (25%). It has been reported (10, 11) that the amplification of the *ERBB2* gene is not correlated with the ER and PR status, but with the number of metastatic nodes. Our results are in agreement with these findings and indicate that *ERBB2* gene overexpression is not correlated either with ER and PR or with pS2 gene expression. On the other hand, a correlation may exist between *ERBB2* gene overexpression and the presence of metastatic nodes (Fig. 5). However, although most *ERBB2*-overexpressing cancers appear to be associated with metastatic nodes, there is a large number of cancers with metastatic nodes that do not exhibit *ERBB2* gene overexpression.

CONCLUSIONS

Our present study establishes pS2 gene expression (mRNA and protein) as an estrogen-dependent biochemical and immunocytochemical marker in breast cancer. Certainly, the use of immunostaining in paraffin-embedded sections offers the opportunity to simultaneously study both the histology and presence of pS2 protein. As for ER ICA and PR ICA (16, 17), this may be very useful when a tumor specimen is so small as to preclude both histological and biochemical examination. In addition the histopathological structure of the paraffin sections is well conserved, and paraffin-embedded sections that have been stored for several years can be used for pS2 protein immunostaining (data not shown), which is a considerable advantage over frozen sections for retrospective inquiries. Thus, the paraffin sections described in this paper appear to be most suitable to reveal estrogen-responsiveness and, therefore, the presence of functional ER in human breast cancers. Moreover, with ER ICA and PR ICA, pS2 ICA should be highly useful for examining tumor heterogeneity with respect to estrogen-responsiveness.

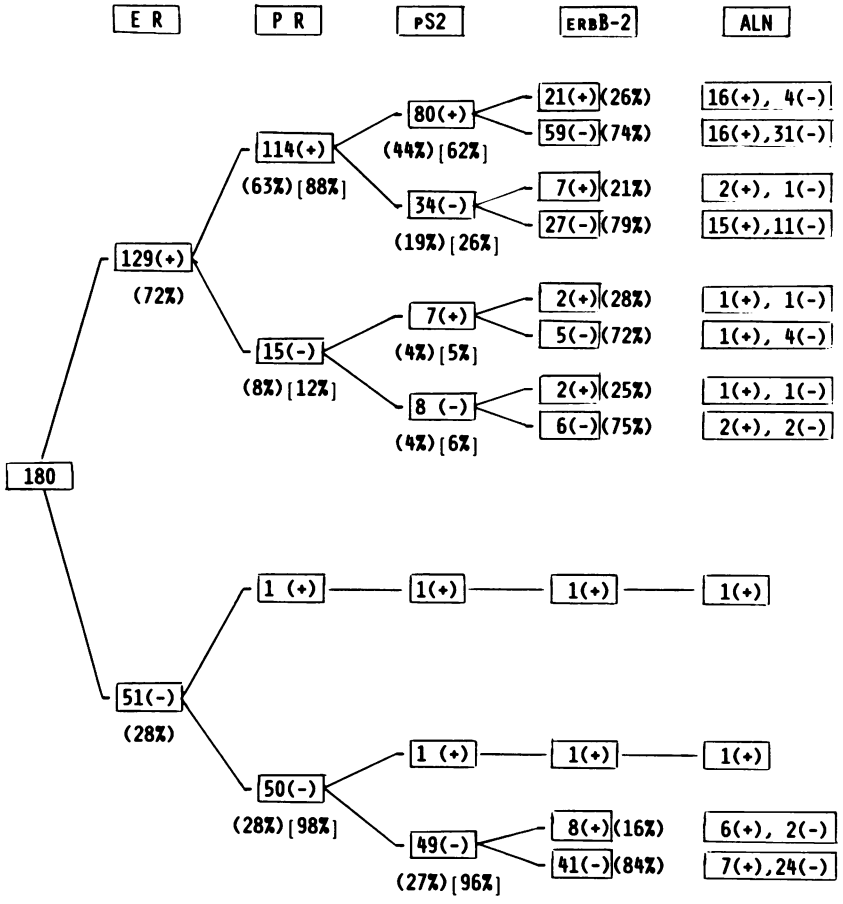

FIG. 5. Expression of the ER, the PR, the pS2 gene, and the *ERBB2* oncogene in 180 breast cancer tumors. ER and PR levels were determined using ER DCC, PR DCC, ER ICA, and PR ICA, except for two of the seven ER-positive, PR-negative, and pS2-positive tumors for which PR was only assayed using PR DCC. pS2 gene expression was determined using both pS2 mRNA analysis and pS2 ICA. Overexpression (positivity) and nondetectable or very low levels of expression (negativity) of the *ERBB2* gene was determined by mRNA analysis. Out of the 80 ER-, PR-, and pS2-positive tumors, 66 were moderate or high (2+ and 3+) in pS2 mRNA and protein, whereas out of the 7 ER-positive, PR-negative, and pS2-positive tumors, 5 were high in pS2 mRNA and protein. The only ER-negative, PR-positive, and pS2-positive tumor was low in pS2 mRNA and protein, whereas the only ER-negative, PR-negative, and pS2-positive tumor was high in pS2 mRNA and protein. The distribution of axillary lymph node metastasis (ALN) is indicated in the right-hand side column [ALN(+) indicate the presence of at least one metastasized node; only cases in which at least three lymph nodes could be examined have been taken into consideration]. Percentage figures in parentheses refer to the total number of tumors (180 tumors), whereas percentage figures in brackets refer to either ER-positive tumors (129 tumors, *Upper*) or ER-negative tumors (51 tumors, *Lower*).

Although screening of pS2 gene expression clearly provides a basis for establishing subclasses of ER-containing tumors, it is unknown at the present time whether pS2 expression, and heterogeneity in pS2 expression, provide additional information concerning the clinical unresponsiveness to hormone therapy seen in ≈40% of patients with ER-positive breast cancers and ≈20% of patients with both ER- and PR-positive breast cancers. In this respect, the existence of a ER-positive, PR-positive, and pS2-negative subclass that represents 30% of ER-, PR-positive breast cancers is most interesting. It defines a subclass, reminiscent of the breast cancer T-47D cell line, which is ER-positive and pS2-negative (5, 18) and has a high level of constitutive estrogen-insensitive PRs (ref. 19 and references therein).

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