Immunocytochemical Localization of Estrogen Receptor in Human Breast Tissue


Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff CF4 4XX, Wales [K. J. W., N. B., D. W. W., K. G., R. I. N.], and Departments of Surgery [J. R., R. W. B.] and Pathology [I. O. E., C. W. E.], City Hospital, Nottingham, England

ABSTRACT

An immunocytochemical assay for the measurement of estrogen receptor (ER-ICA; Abbott Diagnostics) has been evaluated in 163 human breast carcinomas. Specific binding was observed in the nuclei of 111 of 163 (68%) tumors. An excellent correlation was observed between the ER-ICA and the estrogen receptor enzyme immunoassay. A significant relationship was observed between ER-ICA status and percentage of ER-ICA negative cells, histological grade of malignancy, and mitotic activity of the tumors. A significant correlation was also observed between ER-ICA status and age at mastectomy with 50% of patients with ER-ICA positive breast tumors presenting with their disease over 60 years of age. No association was observed with either tumor size or patient nodal status. Examination of the proportion of negative cells within tumors revealed a trend for the acquisition of poor prognostic features to be associated with an increase in the negative cell population. Data on the recurrence free interval of these patients showed a significant recurrence free advantage in ER-ICA positive patients, particularly those whose tumors contained low numbers of negative cells.

INTRODUCTION

Throughout the last 15 years the assessment of the estrogen receptor content of human breast tumors has generated a considerable amount of valuable information relevant to the clinical management of the disease. In patients with systemically advanced breast cancer a good correlation exists between the receptor status of either the primary (1) or metastatic (2) tissue and response to endocrine therapy. Similarly, consideration of the receptor status in association with various clinical and pathological parameters has facilitated the selection of patients most likely to develop an early recurrence after mastectomy (1, 3). Procedures for measuring estrogen receptor however have been associated with certain methodological difficulties (4-7) and have been limited by the requirement to solubilize the receptor prior to the assay. Thus, information concerning the heterogeneity of receptor expression has been confined to using relatively insensitive assays (8) on dissected portions of tumor tissue (9).

New assays are, however, now available which use monoclonal antibodies to directly detect the receptor protein and have been adapted to provide a highly sensitive ER-EIA1 for use on solubilized fractions of breast tissue (10-12) and an ER-ICA which can detect ER expression in individual tumor cells (13-15). The current study reports on our emerging experience with the ER-ICA and demonstrates that a knowledge of the proportion of tumor cells in overtly estrogen receptor positive tumors that do not express significant amounts of estrogen receptor may be of clinical significance.

PATIENTS AND METHODS

Tumor samples were obtained from 163 consecutive patients at the City Hospital in Nottingham under the care of Professor R. W. Blamey. A simple or s.c. mastectomy was undertaken and lymph node biopsy samples were removed from the lower axilla, from the apex of the axilla, and from the internal mammary chain (16). Patients with tumor cells histologically evident in any node were classified as lymph node positive. The menopausal status and age at mastectomy of each patient were recorded in addition to tumor size. Histological grade of malignancy was assessed in all tumors by C. W. E. and I. O. E. using a modification (17) of Bloom and Richardson's criteria. Tumors were classified I to III with increasing loss of differentiation. The mitotic activity of tumors was assessed by counting the number of mitotic figures in 10 or 20 high power fields at the peripheral infiltrating margin of the tumor. Divisions I-III corresponded to 0-9, 10-19, and over 20 mitotic figures/10 high power fields, respectively.

All primary breast cancer patients were followed up at 3-month intervals for 18 months and at 6-monthly intervals thereafter. Treatment was withheld until recurrence; no patient received any form of systemic adjuvant therapy.

Receptor Analysis

Immediately after surgery tumor tissue was snap-frozen and stored in liquid nitrogen before transportation in dry ice to the Tenovus Institute. Samples were stored at −70°C until assay.

Assay Procedures

Estrogen Receptor Immunocytochemical Assay. The immunocytochemical detection of ER was carried out using an assay kit developed by Abbott Diagnostics (Abbott Laboratories, North Chicago, IL). The assay uses a sensitive PAP technique for the visualization of estrogen receptors in frozen tissue sections. Cryostat sections (5 µm) were mounted on slides treated with tissue adhesive. Tissues were fixed by placing them in 3.7% formaldehyde-phosphate buffer solution for 15 min. The slides were transferred to a 10 mm PBS (pH 7.2-7.4) bath for 5 min, followed by methanol at −10°C to −25°C for 5 min and acetone at −10°C to −25°C for 3 min. The slides were then rinsed in PBS for 5 min and stored in specimen storage medium (0.25 M sucrose and 6.9 mM magnesium chloride) in 250 ml PBS plus 250 ml glycerol at −10°C to −20°C for up to 1 month before assay. With the exception of the methanol and acetone fixation all PBS washes and incubations were conducted at room temperature.

Stored sections were washed in PBS prior to the ER-ICA and then incubated for 15 min with normal goat serum (blocking agent). The primary antibody (anti-human ER rat monoclonal antibody H222 Spy) was added dropwise to one section of each specimen, control antibody (rat IgG) was added to a second section from the same specimen, and sections were further incubated for 30 min. Slides were washed twice in PBS for 5 min before incubation with the bridging antibody (goat anti-rat IgG) for 30 min. Slides were washed twice in PBS for 5 min before and after a further 30-min incubation with rat PAP complex. A chromogen substrate solution containing hydrogen peroxide (0.06%) and diaminobenzidine-4HCl was added to each specimen for 6 min. The reaction of peroxidase in the PAP complex with hydrogen peroxide converts the diaminobenzidine-4HCl to an insoluble reddish brown product. Sections were immersed in distilled water before counterstaining with Harris' hematoxylin (1% v/v) for 6 min. Sections were rinsed in tap water for 5 min, dehydrated in alcohols, cleared in xylene, and mounted under coverslips in dibutylphthalate xylene solution.

All specimen evaluation was performed on an Olympus microscope.
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Fig. 1. Immunocytochemical localization of ER in breast tumors using an anti-human ER rat monoclonal antibody (H222). Tumors exhibiting decreasing intensity of stain and increasing proportions of ER-ICA negative cells are illustrated.

(BH-2) using ocular magnifications of x40. Control slides were checked for nonspecific binding before assessing the percentage of tumor cells stained by the primary antibody. Quantitative assessments of the number of positively stained cells in 60 tumors were made by using a 25-point graticule. For each tumor a total of 30 fields were counted and a mean percentage staining figure was calculated. The same tumors were then evaluated by two personnel (K. J. W. and R. I. N.) using a dual-viewing attachment to the Olympus microscope. Comparison of the results obtained using both methods of assessment demonstrated excellent agreement ($r = 0.82, P < 0.01$). The latter method was used throughout the study. Tumors were classified as ER-ICA positive where greater than 2% of tumor cells were stained positively. The intensity of staining in target cells was assessed semiquantitatively using a scoring index of 0 to 3 corresponding to negative, weak, intermediate, and strong staining intensity. Using this system tumors were classified into 3 groups: those which did not contain any positive cells (0); those which contained cells expressing weak and intermediate levels of receptor expression (1+, 2+); and those containing cells expressing all 3 categories of receptor expression (1+, 2+, 3+). Areas of normal or benign breast tissue were excluded from the final assessment.

Estrogen Receptor Enzyme Immunoassay. The enzyme immunoassay for the detection of ER was carried out using an assay kit developed by Abbott Diagnostics (Abbott Laboratories). The assay is a solid phase immunoassay based on the “sandwich” principle (18) as described previously (12).

Statistical Analysis

Data were analyzed using a $\chi^2$ statistic for contingency table analysis and $\chi^2$ for trend according to the work of Armitage (19). Recurrence free interval was assessed by life table analysis (19).

RESULTS

Localization of Estrogen Receptors in Breast Cancer Specimens

The immunocytochemical localization of ER in breast tumors using an anti-human ER rat monoclonal antibody (H222) revealed specific binding in the nuclei of 111 of 163 (68%) tumors. Both the proportion of tumor nuclei expressing the antigen and intensity of stain were, however, highly variable (Fig. 1). No binding was observed in the cytoplasm of the tumor cells, in either stromal or muscle components of the breast tumors, or in blood vessels or infiltrating macrophages. Antibody binding was sometimes observed in the nuclei of normal and benign components of the breast tumor but was not included in the analysis.

Association of ER-ICA with Various Clinical, Pathological, and Biochemical Features of Breast Cancer

ER-EIA. An excellent correlation was observed between the ER immunocytochemical and enzyme immunoassays in the 163 breast tumors (Fig. 2). Thus, while 108 of 111 ER-ICA positive tumors were also positive by the ER-EIA, with a mean receptor value of $213 \pm 172$ fmol/mg protein, only 15 of 52 ER-ICA negative tumors contained ER-EIA values above 10 fmol/mg protein. Indeed, the mean ER-EIA value in this group was considerably lower (19 fmol/mg protein) than that observed in double positive samples.

Menopausal Status and Age at Mastectomy. A significant correlation also exists between ER-ICA status and age at mas-
elderly patients (>60 years), with only 4 and 10% of women for this age group in women with ER-ICA negative tumors was between the ages of 25 and 40 years, the corresponding value of menopausal status of women at the time of presentation of their disease (not illustrated). In general, tumors from postmenopausal women were more likely to be ER-ICA positive, to possess cells expressing high concentrations of ER, and to contain fewer ER-ICA negative cells than their premenopausal counterparts.

Histological Grade of Malignancy. The data presented in Fig. 4 clearly demonstrate that a relationship exists between the histological grade of malignancy of breast tumors and their ER-ICA status. Thus while over 75% of ER-ICA negative primary breast tumors were poorly differentiated Grade III carcinomas, the corresponding value for ER-ICA positive samples was 31%.

The relationship between ER-ICA status and grade of malignancy was independent of the menopausal status of the patient (Fig. 4a-b). Examination of the individual components of tumor grade and ER-ICA status showed an association between these parameters, with ER-ICA negativity being more often observed in tumors with high rates of mitotic activity (Fig. 4d), increased nuclear pleomorphism (Fig. 4e), and lack of tubular differentiation (Fig. 4f). These associations were independent of menopausal status of the patients (not illustrated).

Although the subdivision of the ER-ICA positive tumors according to the semiquantitative level of ER expression (1+2+3+ or 1+2+) did not reveal any additional association with either the histological grade of malignancy of breast tumors or its individual components (Fig. 5), a relationship was observed with the percentage of ER-ICA negative cells in ER-ICA positive tumors and histological grade of malignancy (Fig. 6a). Indeed, while well differentiated Grade I tumors comprised approximately one-third of ER-ICA positive tumors containing <50% negative cells, the value fell to <20% in tumors containing >50–98% ER-ICA negative tumor cells and to <10% in ER-ICA negative samples. These parameters were again independent of the menopausal status of the patient. A similar relationship was observed with mitotic activity, with over 50% of ER-ICA positive tumors containing <50% negative cells showing low numbers of mitotic figures (Fig. 6d). No association was determined between the percentage of ER-ICA negative cells in positive tumors and nuclear pleomorphism (Fig. 6e) or tubular differentiation (Fig. 6f).

Tumor Size and Lymph Node Status. No significant association was observed between either tumor size or lymph node status and any of the above parameters, although ER-ICA negative tumors may contain a higher proportion of >3 cm tumors (Fig. 7).

Recurrence Free Interval. Examination of the recurrence free interval of patients following mastectomy showed that women with ER-ICA positive tumors had a more favorable early prognosis than those with ER-ICA negative disease (Fig. 8a). Further stratification of the data according to the proportion of negative cells within ER-ICA positive breast tumors, however, identifies a high rate of recurrence in patients whose tumors...
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Fig. 4. Relationship between ER-ICA status and histological grade of malignancy in (a) all patients, (b) premenopausal patients, and (c) postmenopausal patients. The individual components of tumor grade (d), mitotic activity (e), nuclear pleomorphism, and (f) tubular differentiation are also presented in relation to ER-ICA status. , , , and , increasing loss of differentiation (a, b, c, f), increasing nuclear pleomorphism (e), and increasing numbers of mitotic figures (d) in breast tumors. Number of tumors in each category on right of each column. For b, e, and f, there were insufficient numbers of tumors to perform a statistical analysis.

Fig. 6. Relationship between percentage of ER-ICA negative cells and histological grade of malignancy. (a) All patients, (b) premenopausal patients, (c) postmenopausal patients. The individual components of tumor grade (d), mitotic activity (e), nuclear pleomorphism, and (f) tubular differentiation are also presented in relation to ER-ICA status. , , , and , increasing loss of differentiation (a, b, c, f), increasing nuclear pleomorphism (e), and increasing numbers of mitotic figures (d) in breast tumors. Number of tumors in each category on right of each column. For b, c, e, and f, there were insufficient numbers of tumors to perform a statistical analysis.

Fig. 5. Relationship between intensity of ER-ICA staining and (a) histological grade of malignancy, (b) mitotic activity, (c) nuclear pleomorphism, and (d) tubular differentiation. , , , and , increasing loss of differentiation (a, d), increasing nuclear pleomorphism (c), and increasing numbers of mitotic figures (b) in breast tumors. Number of tumors in each category on right of each column. For a, b, c, and d, \( \chi^2 \) analysis was not significant.

Fig. 7. Relationship between ER-ICA status, staining intensity, and percentage of ER-ICA negative cells and (a-c) tumor size (, <2 cm; , 2.1-3 cm; , >3 cm) and (d-f) patient lymph node status at mastectomy (, node positive; , node negative). Number of tumors in each category is on right of each column. \( \chi^2 \) analysis revealed no significant associations between these parameters.

DISCUSSION

Several reports have now established in breast cancer specimens a relationship between ER expression as determined by the steroid binding and immunocytochemical assays (12-14, 20, 21). Although in general a good correlation has been achieved between these assays, the immunocytochemical procedure has several distinct advantages in that it may be performed on small biopsy samples (13, 14) or breast tumor aspirates (22, 23). Moreover, it also provides information on the heterogeneity of ER expression, not only between the tumor
and its normal and benign components but also within the tumor cell population (13, 14, 21). The current paper extends these observations to demonstrate that the ER-ICA status of breast cancer also relates to the breast tumor ER-EIA status and level (Fig. 2), the histological grade of malignancy of breast cancers (Figs. 4, a–c), and the individual components of grade (Fig. 4, d–f), with ER-ICA negative tumors tending to arise in younger women with more poorly differentiated ER-ICA negative tumors. No strong association was, however, observed between ER-ICA status and either tumor size or lymph node status, two important prognostic variables in breast cancer. These data parallel previous observations from this laboratory regarding the relationship between the above parameters and ER status as determined by either an enzyme immunoassay (12) or a steroid binding assay (24, 25).

In the future it is likely, however, that the most important feature of immunocytochemistry lies in its ability to provide information on the distribution of hormone receptors within cell populations. Clearly, Fig. 1 demonstrates that ER expression is a highly variable parameter with the intensity of stain and the distribution of positive and negative cells differing extensively between tumors. Although there are many methodological variables that may influence these parameters (storage and processing of tissues, etc.) the results obtained in the present study suggest an underlying physiological basis. For example high levels of ER expression, as determined by the ER-ICA (i.e., presence of 3+ cells), were most often observed in elderly patients, a result in keeping with the known influence of age on receptor levels (3, 25). Furthermore, it can also now be seen that the tumors from these patients are more likely to contain a greater proportion of cells expressing detectable amounts of ER. Conversely, the known association of ER status and histological grade of malignancy can now be extended to include the observation that ER positive tumors that contain <50% apparently negative cells are more likely to be well differentiated than ER-ICA positive tumors which contain a high proportion of negative cells. Significantly, a decreased number of negative cells in positive tumors also appears to be associated with a more favorable prognosis with this group of patients showing fewer earlier relapses after mastectomy (Fig. 8).

It is difficult to currently envisage the extent to which these data may influence the future management of breast cancer patients. However, it seems likely that a knowledge of the proportion of negative cells within an ER-ICA positive tumor may be of both prognostic and therapeutic importance. Indeed, the relationship observed between high numbers of ER-ICA negative cells and the poor prognostic features of high numbers of mitotic figures and poorly differentiated tumor types suggests that these patients might derive benefit from combined endocrine and cytotoxic therapies. Caution, however, should be used in interpreting the authenticity of negative cells within a tumor population. Two possible explanations exist. Firstly, the cells may be truly autonomous, a suggestion that is supported by the data relating to the pathological features of the tumor. Alternatively, the detection of ER-ICA negative cells may result from an inability of the assay to detect low levels of ER. Indeed, ER-ICA negative cells are often observed in normal breast tissue and are also present in increased numbers in breast tumors removed from premenopausal women. These data are suggestive that tissues may remain hormone sensitive and yet have insufficient receptor to be detected by the ER-ICA. Studies to resolve these differences are currently in progress.

REFERENCES


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Kerry J. Walker, Nasser Bouzbar, John Robertson, et al.


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