Immunocytochemical Analysis of Estrogen Receptors as a Predictor of Prognosis in Breast Cancer Patients: Comparison with Quantitative Biochemical Methods

Laura B. Kinsel, Eva Szabo, Geoffrey L. Greene, John Konrath, George S. Leight, and Kenneth S. McCarty, Jr.

Duke University Medical Center, Durham, NC 27710; Bellevue Medical Center, New York, NY; University of Chicago, Chicago, IL

ABSTRACT

Biochemical quantitation of estrogen receptors has been used to predict prognosis in breast cancer. Immunocytochemical analysis of estrogen receptors correlates with biochemical analysis but has very few follow-up studies in the literature to validate it as a prognostic indicator. 257 patients were followed for up to 10 years (median, 6.2 years) after primary surgical treatment. Estrogen receptor analysis using both biochemical and immunocytochemical techniques was performed on their tumor specimens. Patients with positive estrogen receptor values had longer survival than patients with negative values. This was demonstrated by both methods in the first 5 years of follow-up but only by immunocytochemistry after 5 years. The relationship between estrogen receptor status and disease-free interval was less strong than with survival. This study demonstrates that immunocytochemical estrogen receptor analysis was of prognostic significance.

INTRODUCTION

Quantitation of ER using ligand-binding (biochemical) assays has been used as a predictor of prognosis and response to endocrine therapy in breast cancer patients for over 10 years. In the many studies examining the issue of the value of ER in estimating prognosis, the relationship between ER status and survival has tended to be stronger than that between ER and DPI. These relationships were apparent in short term studies (less than 3 years of follow-up) yet were markedly diminished in long term studies (follow-up) of 5 years or more (1-9).

Because of the inability of biochemical ER measurements to assess intratumoral heterogeneity, histochemical techniques with high sensitivity and specificity have been sought. With the purification of ER and the production of monoclonal antibodies to the ER protein, an immunocytochemical assay (ER-ICA) has been developed (10). It has been shown to correlate with biochemical techniques in studies from many different centers (11-19). However, few studies have addressed its prognostic significance beyond three years (20-21). Because of the need for more clinical studies to validate this assay, we have studied a cohort of 257 breast cancer patients for up to 10 years with median follow-up of 6.2 years. Estrogen receptor levels, measured by biochemical methods, were compared to the ER-ICA results in their ability to predict disease-free interval and survival in this cohort.

MATERIALS AND METHODS

Patient Population

The study group consisted of 257 patients treated at Duke University Medical Center or Cabarrus Memorial Hospital from 1976 to 1980 for breast cancer whose primary tumor specimens were sequentially accessioned in the Endocrine Oncology Laboratory. Only those patients with sufficient cancerous tissue for immunocytochemical analysis were included in the study. Both pre- and postmenopausal patients were included.

Clinical Data

Parameters recorded at the time of diagnosis included age, menopausal status, race and stage using the following system: Stage 1, no positive axillary nodes, no skin or muscle involvement; Stage 2, 1-3 positive nodes, no skin or muscle involvement; Stage 3, 4+ positive nodes and/or skin or muscle involvement; Stage 4, distant metastases.

Of the 257 patients, 166 patients received modified radical mastectomies as their primary surgical treatment whereas 51 received radical mastectomies, 14 received simple mastectomies without radiation. The remaining patients received alternate primary therapies. Sixty-five patients received adjuvant therapy. Twenty-nine patients had metachronous cancer in the contralateral breast.

Estrogen Receptor Analyses

Tissue Preparation. Tissues were quick frozen after being washed in 0.005 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-0.01 M Tris-0.0015 M EDTA-0.01 M thyroglobulin-0.02% NaN3 buffer (pH 7.4) and were maintained at -70°C in airtight light nitrogen capsules from the time of excision until sectioning for immunocytochemical analysis.

Quantitative (Biochemical) Methods. Cytosol estrogen receptor analysis was performed using multiconcentration dextran-coated charcoal or sucrose gradient binding assays at the time tissue was first obtained as previously described (22). In brief, 3H-hexa-labeled estradiol (New England Nuclear) was used as the radiolabeled ligand for each assay and nonspecific binding was reduced in the cytosol by incubation with 100-fmol concentrations of dihydrotestosterone. Parallel control incubations of cytosol were also performed with 250-fold concentrations of nonlabeled diethylstilbestrol in addition to radiolabeled hormone. Sucrose density gradient analysis was performed with 250 µg/liter cytosol incubated for 4 h at 4°C with 1.6 pmol radiolabeled ligand while dextran-coated charcoal analysis was performed after 16-h incubation at 4°C with concentrations ranging from 1.6 to 0.0125 pmol of radiolabeled ligand. Data from each assay were analyzed as described previously (22) and results expressed as femtomoles (fmol) of estrogen binding per milligram of cytosol protein. Results greater than or equal to 10 fmol/mg protein were considered positive.

Immunocytochemical Method. Serial cryostat sections were fixed in 33.7% formaldehyde-140 mM NaCl, buffered with 0.1 M phosphate, pH 7.4, at 25°C for 10 min, followed by immersion in 100% methanol for 4 min and acetone for 1 min at -10°C to -25°C. The peroxidase-antiperoxidase method for immunocytochemical localization was performed as previously described (12). Single cross-sectional slides of each tumor were used for ER-ICA localization. Normal goat serum was used as the blocking agent. The primary antibody H222 (anti-ER) was used at a concentration of 5.0 µg/ml. The bridging antibody was goat anti-rat immunoglobulin and the peroxidase-antiperoxidase complex was of rat origin. Control slides consisted of sections of cancers adjacent to those stained with the primary antibody for which normal rat serum was used in place of the primary antibody. The slides were treated poly-L-lysine to improve adhesion of tissue. Localization of antigen-antibody complexes with peroxidase was developed using dianisidobenzidine-H2O2.

Immunocytochemical localization of estrogen receptors was scored in a semiquantitative fashion incorporating both the intensity and the...
distribution of specific staining. The evaluations were recorded as percentages of positively stained target cells in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), and 3+ (strong, with minimal light transmission through stained nuclei). For each tissue, a value designated as the HSCORE was derived by summing percentages of cells stained at each intensity multiplied by the weighted intensity of staining:

$$HSCORE = \sum P_i (i + 1)$$

When $i = 1, 2, 3,$ and $P_i$ varied from 0 to 100%. An HSCORE of 75 or greater was considered positive.

Cellularity. The cellularity of the tumor specimens was determined by estimation of the percentage of malignant epithelium in hematoxylin & eosin-stained sections, adjacent to the sections used for immunocytochemical analysis.

Histological Evaluation. Histological and nuclear grade were determined as previously described (12).

Data Analysis. Clinical parameters, biochemical and immunocytochemical assay values were coded separately in a blinded fashion and maintained as independent files until completion of the study. Statistical analysis of biochemical and immunocytochemical values, and clinical data was performed using the Cox-Mantel test and the Kaplan-Meier Survival estimate. Intra- and interobserver reliability and comparison of ER-ICA with the biochemical ER values were assessed using the Pearson correlation coefficient. Sensitivity, specificity, and accuracy were determined using the qualitative biochemical ER value as the standard. Immunocytochemical assay results were each classified as true positive (TP), true negative (TN), false positive (FP), or false negative (FN) in relation to the biochemical value. The formula used for sensitivity, specificity, and accuracy are as follows (23):

- Sensitivity = TP/(TP + FN)
- Specificity = TN/(TN + FP)
- Accuracy = (TP + TN)/(TP + TN + FP + FN)

The threshold of HSCORE ≥ 75 being considered positive was determined previously, maximizing sensitivity and specificity in a learning population and validated with a separate and distinct test population (11–12).

RESULTS

Immunocytochemical staining of estrogen receptors was observed only in the nucleus of target cells. Control slides had for each tumor section minimal background staining. Intraobserver reliability was $r = 0.94$, whereas interobserver reliability was $r = 0.91$. Correlations were observed between the log of the biochemical ER value and ER-ICA HSCORE ($r = 0.63, P < 0.001$). When sensitivity, specificity, and accuracy were determined by comparing ER-ICA with biochemical ER value (using the thresholds of HSCORE ≥ 75 being positive and biochemical ER ≥ 10 fmol/mg protein being positive), there was an overall agreement of 69% (accuracy) with sensitivity and specificity of 52% and 88%. There was not a significant relationship between ER, determined by either method, and tumor cellularity (ER-ICA, $r = -0.116$; log biochemical ER, $r = 0.027$).

The relationships between ER status, disease-free interval (DFI) and survival are shown in Figs. 1 and 2 for both biochemical and ICA methods. There is significantly longer survival in patients with positive ER-ICA value than those with negative ER-ICA (Fig. 1B, $P < 0.005$). This difference does not persist beyond 5 years for the biochemical assay (Fig. 1A, $P = 0.986$). The relationship between ER status and DFI is less strong than with survival. There is initially a difference in the incidence of metastatic disease at diagnosis between ER-positive and -negative groups for both methods. However, this difference is maintained for 10 years using ER-ICA, though not statistically significant (Fig. 2B, $P = 0.12$) but not maintained after 3.5 years using the biochemical ER value (Fig. 2A, $P = 0.11$).

When separating patients by stage, it was found that the difference between ER-positive and -negative patients in DFI using Fig. 3 ER-ICA is well demonstrated in Stages 2 and 3 (Fig. 3, A and B). By definition, patients with distant metastases at the time of diagnosis have a DFI of 0, therefore this group was not included in this analysis. There was a difference favoring ER-ICA-positive versus -negative groups in survival for Stages 1–3 (no positive nodes, and one or more positive nodes or skin/muscle involvement). Survival curves for Stage 2 and 3 disease are shown in Fig. 4B. There was no difference in survival between ER-ICA ($P = 0.03$, Fig. 4A) probably relating to the lower number of cases used for each group. There were no notable differences between ER groups using the biochemical method except for a slight difference in survival in Stage 2 patients.

Analysis of survival with DFI was performed after separating cases by both histological and nuclear differentiation and by

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Fig. 1. A, survival from date of primary treatment is shown for the entire population of 257 patients. Estrogen receptor determined biochemically was considered positive if a value ≥10 fm/mg protein as observed (dashed line). 123 patients were in this category. 134 patients had biochemical estrogen receptor <10 fm/mg of protein. The convergence of the lines can be appreciated at approximately 5 years. B, survival from date of primary treatment for full study population for immunocytochemically determined receptor positive as compared to immunocytochemically determined receptor negative patients. The receptor was considered positive if a HSCORE of ≥75 was observed. There are 139 patients in the group considered receptor positive by ICA. 118 patients had ICA HSCORES of <75. The separation of these groups is apparent throughout the period of followup ($P = 0.0048$).
Fig. 2. A, disease free interval comparisons for biochemically determined estrogen receptor positive as contrasted to estrogen receptor negative patients. The disease free interval for the entire population of 257 patients is shown. ER-positive lesions are represented by a dash line (123 patients). Those with ER values of 10/fm/mg protein are represented by a solid line (134 patients). The crossover of these lines is apparent at approximately the third year. B, disease-free interval comparison for immunocytochemically determined estrogen receptor-positive versus immunocytochemically determined estrogen receptor-negative patients. The complete population of 257 patients are represented. The disease-free interval for HSCORE positive (>75) is shown with a dashed line (118 patients). The disease free interval for the immunocytochemically determined estrogen receptor negative patients is shown with a solid line (139 patients). A separation is seen throughout the period of follow-up, although statistical significance is not achieved throughout this period. The maximum separation is noted in the first 5 years.

Fig. 3. A, survival comparison for patients with Stage 2 disease (one to three positive nodes) in the initial surgical specimen based on separation of populations by immunocytochemically determined estrogen receptor negative. A comparison of disease-free interval for 37 patients whose initial specimen showed one to three nodes involved with tumor is shown separated according to estrogen receptor-ICA positive (HSCORE ≥ 75) as indicated by the broken line (25 patients) as contrasted to ER-ICA-negative patients as indicated by the solid line (12 patients). The separation of these groups approach statistical significance despite the small number of patients in this subset. B, comparison of disease-free interval for ER-ICA-positive versus ER-ICA-negative patients whose primary breast resection was associated with four or more nodes positive or skin or muscle involvement (Stage 3). A comparison of the disease free interval for 74 patients with four or more nodes positive at the time of their initial primary treatment is shown. ER-ICA-positive patients are depicted by a dashed line (42 patients) while ER-ICA-negative patients are shown by a solid line (32 patients). The separation of the disease free interval advantage is apparent through 5 years with convergence thereafter.

ER status, as determined by ER-ICA and biochemical methods. Among intraductal lesions no significant differences in survival or DFI were observed beyond 3 to 4 years. When using ER-ICA and all lesions had negative biochemical ER status. All histologically well-differentiated lesions were ER-positive by both methods. However, among patients with moderately differentiated lesions, ER-ICA status was a significant prognostic factor (Fig. 5A) with enhanced survival for ER-ICA-positive lesions. Although early DFI cures favored ER-ICA-positive lesions, the difference was not maintained beyond 6 years (P = 0.63, data not shown). In contrast, patients with biochemical ER-negative moderately differentiated lesions had a trend towards enhanced survival and DFI (data not shown). A similar trend for improved survival and DFI, was observed for ER-ICA-positive lesions (Fig. 5B). Although ER-ICA-positive lesions had enhanced early survival and DFI, the differences were not maintained beyond 6–8 years (P = 0.27 and 0.34 for survival and DFI, respectively). In this group, biochemical ER status was not a prognostic factor beyond 3.5–5 years. (P = 0.93 and 0.60 for survival and DFI, respectively, data not shown). When lesions were stratified by nuclear grade, a similar trend was observed; classification by ER-ICA status had more prognostic importance than biochemical ER status in predicting survival and DFI, except in lesions with well differentiated (grade 3) nuclear features where all lesions were receptor positive by both methods. Due to small numbers of patients within each category survival and DFI curves did not reach statistical significance, except in lesions with moderately differentiated (grade 2A) nuclei where the survival advantage for ER-ICA positive tumors was maintained throughout the study interval (P = 0.03, data not shown).
ER-ICA AS A PREDICTOR OF SURVIVAL AND DFI

DISCUSSION

ICA has been shown to be a sensitive, specific, and reproducible method for detecting estrogen receptors in breast cancer cells. It correlates with biochemical analysis reasonably well but is not equivalent (11–19). ICA provides a unique perspective to ER analysis in that it is able to determine the distribution of receptors in heterogeneous tissues and assure that the receptors being evaluated reside in the malignant tissue and not benign or normal structures.

Biochemical analysis of ER in cytosol extracts has been noted to be a predictor of disease-free interval (1–3) and survival (1–8). However, most of the studies showing significant differences in DFI between ER-positive and -negative groups have had median follow-up of less than 3 years (1–3). Of the studies with 5 or more years of follow-up, most have shown that there is no difference in DFI between groups or show that ER-positive patients begin to recur more often than ER-negative patients after 3 years (4–8). Some found no difference at any point in time (9). While most studies have found differences in survival between groups, there are reports of no difference existing between groups at any time in a 10-year study period (9). In the present study, though ER-positive patients initially had better disease-free intervals than ER-negative patients, they were found to have worse DFIs than ER-negative patients after 3 years. A similar crossover occurred for survival after 5 years. Thus, despite initially good predictive value in the early years of follow-up, ER, as determined by biochemical methods, could not reliably predict prognosis for the longer term.

Immunocytochemical analysis of ER, however, was found to predict survival and DFI through the full 10 years in this study. The other prognosis study of ER-ICA only reported 3 years of follow-up and found significantly longer survival and DFI in ER-positive patients than in ER-negative patients by both ICA and biochemical methods (21). However, in this same study, use of the Cox Proportional Hazards model revealed that ER-ICA was the single most important predictor of survival (21).
Why ICA appears to have better predictive value for prognosis than biochemical analysis is not clear. Biochemical analysis has been shown in this laboratory to have relatively higher values than ICA in poorly differentiated (grade 3) tumors and lower values than ICA in well-differentiated (grade 1) tumors. Also, biochemical analysis was found to have more of its negative values in the borderline range than immunocytochemistry. This may result from the contribution of non-receptor steroid binding proteins in ligand binding assays or a contribution of low levels of binding by nonmalignant tissue. It had been suggested that the protein of nontumor elements may dilute the resulting ER value (24). Immunocytochemistry does not have this problem since it allows the evaluation of malignant components alone. However, this study demonstrates that neither method is significantly affected by the relative amount of tumor in the specimen used. Therefore, this does not provide a sufficient explanation for the difference in performance between methods. ICA has potential bias which biochemical analysis lacks. The observer can easily see the levels of differentiation of the tumor by the histological pattern of the stained nuclei while scoring slides. Examination of survival after separating by histological and nuclear grade demonstrates that the observations found in this study are not explained by this bias. Estrogen receptor status still predicts survival (Fig. 5, A and B). More studies are needed to confirm the difference between methods and evaluate the differences in technique which may be responsible for this phenomenon.

Surgical stage has been shown to be one of the most important predictors of prognosis in breast cancer patients (25). When patients are divided into groups by stage, ER-ICA is still able to predict prognosis via DFI for Stages 2 and 3 and via survival for Stages 1–3. The lack of predictive value for DFI in Stage 1 may relate to the fact that such a high proportion of Stage 1 patients are cured by their primary treatment regardless of ER status. Stage 4 patients initially show a difference in survival between ER-ICA groups but prognosis becomes equally bleak for both groups after 2 years.

Despite the good performance demonstrated for ER-ICA in this study, the technique has some inherent difficulties which must be noted. It is a semiquantitative measure of ER. At present, scoring of slides is subjective and requires that the observer be trained in histopathology. Because it detects ER by its antigenicity, it does not assure that the receptors are functional. Therefore, concomitant use of biochemical techniques assures that a quantitative measurement of receptor activity is included in the evaluation. Progesterone receptor analysis, using both biochemical and ICA techniques, are expected to be of additional benefit (26). Studies examining these analyses and their value in predicting prognosis and response to hormone therapy are in progress.

REFERENCES

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