Bimodal Frequency Distribution of Estrogen Receptor Immunohistochemical Staining Results in Breast Cancer
An Analysis of 825 Cases
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Key Words: Breast cancer; Estrogen receptor; Immunohistochemistry

Abstract

Immunohistochemical analysis is used routinely to determine the estrogen receptor (ER) status of breast cancers in paraffin sections. However, lack of standardization has raised concerns that weakly ER+ tumors often are classified erroneously as ER−. To determine the frequency of weakly ER+ tumors, we reviewed ER immunostains of 825 breast cancers. For each case, we estimated the proportion of ER+ tumor cells and also determined an Allred score (which results in scores of 0 or 2 through 8, based on staining intensity and proportion of positive cells). In 817 cases (99.0%), tumor cells showed complete absence of staining or staining in 70% or more of the cells. Similarly, 818 cases (99.2%) exhibited Allred scores of 0 or of 7 or 8. Thus, with the immunohistochemical method used in our laboratory, ER staining is essentially bimodal. The overwhelming majority of breast cancers are either completely ER− or unambiguously ER+, and cases with weak ER immunostaining are rare.

The use of immunohistochemical analysis to assess the estrogen receptor (ER) status of breast cancers in paraffin sections is now a routine part of pathology practice worldwide. Although ER status as determined by immunohistochemical analysis has been shown to be a prognostic factor for patients with breast cancer, the major goal of determining ER status in current clinical practice is to assess the likelihood of response to hormonal therapy. In this regard, several studies have indicated that ER by immunohistochemical analysis is not only predictive of response to endocrine therapy but also that its ability to predict such responses is superior to that of ER status as determined by ligand-binding assays.

Despite the widespread use of this procedure, the lack of standardization of methods, scoring, and threshold for ER positivity has raised concerns that a substantial minority of patients is being misclassified with regard to the ER status of their tumors when immunohistochemical analysis performed on paraffin sections is used for this purpose. There has been particular concern that weakly ER+ tumors are erroneously being categorized as ER− and that this in turn results in such patients being denied potentially beneficial antiestrogen therapy. It has been our experience that weakly ER+ tumors with the ER immunohistochemical method used in our laboratory are distinctly uncommon. To address this issue in a formal manner, we reviewed all ER immunohistochemical stains performed in our laboratory during a 2-year period. The results of this analysis indicated that in more than 99% of the cases we studied, the ER staining results were completely negative or unequivocally positive and that weakly positive cases are encountered only infrequently.
Materials and Methods

Study Population

We reviewed the accession log in the Beth Israel Deaconess Medical Center (Boston, MA) diagnostic immunohistochemical laboratory to identify all primary invasive breast cancers in which ER immunohistochemical analysis had been performed during the 2-year period between April 1, 1999, and March 31, 2001. A total of 861 such cases was identified. The ER immunohistochemical stains of all 861 cases initially were reviewed by one of us (M.L.B.). During the course of this review, we identified 36 cancers in which the tumor cells were ER– but in which there was no normal breast epithelium on the ER-stained slide to serve as an internal positive control. Given that it is not possible in such cases to determine whether the negative results are true-negative or false-negative results, these were excluded from further analysis. Therefore, the final study population consisted of 825 cases.

The vast majority of cases (784 [95.0%]) were in-house cases; in the remaining 41 cases (5.0%), ER immunohistochemical staining was performed on slides cut from blocks received from outside institutions. In-house core needle biopsy specimens had been fixed in neutral buffered formalin, whereas in-house excision and mastectomy specimens had been fixed in alcoholic formalin. No information was available about the fixatives used for the outside cases.

ER Immunohistochemical Analysis

Immunohistochemical analysis for ER was performed on paraffin sections as part of the routine clinical evaluation of these cases. Tissue sections were deparaffinized in two 5-minute changes of xylene and were rehydrated through graded alcohols to distilled water. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 10 minutes. Subsequently, sections were subjected to heat-induced epitope retrieval by heating in a vegetable steamer in citrate buffer (pH 6.0) for 30 minutes. Following heat-induced epitope retrieval, the primary monoclonal anti-ER antibody was applied to the sections for 1 hour at room temperature (clone 1D5, dilution 1:400; DakoCytomation, Carpinteria, CA). The slides then were incubated sequentially with biotinylated horse antimouse immunoglobulin (Vector, Burlingame, CA) at a 1:250 dilution for 1 hour and streptavidin-horseradish peroxidase (Vectastain Elite ABC, Vector) at a 1:200 dilution for 1 hour. After application of 3,3′-diaminobenzadine (Zymed, San Francisco, CA), the slides were placed in 0.5% copper sulfate in 0.9% sodium chloride for 5 minutes for signal enhancement and then lightly counterstained with hematoxylin.

A positive control sample consisting of an invasive breast cancer known to express ER was included in each staining run. Negative controls in which the primary monoclonal anti-ER antibody was replaced by mouse myeloma protein were performed for each case.

Scoring of ER Immunostains

For each ER immunohistochemically stained slide, we visually estimated the percentage of tumor cells showing nuclear immunoreactivity for ER. In addition, we determined for each case an Allred score, which is a semiquantitative system that takes into consideration the proportion of positive cells (scored on a 0-5 scale) and staining intensity (scored on a 0-3 scale). The proportion and intensity scores are then summed to produce total scores of 0 or 2 through 8.

Results

Among the 825 cases included in this study, ER immunostains were performed on core needle biopsy specimens in 327 (39.6%) and on sections of excision or mastectomy specimens in 498 (60.4%). Of these cancers, 585 (70.9%) were infiltrating ductal carcinomas, not otherwise specified; 92 (11.2%) were infiltrating lobular carcinomas; 105 (12.7%) were infiltrating carcinomas with both ductal and lobular features; and the remaining 43 cases (5.2%) consisted of a variety of other histologic types (including tubular, mucinous, invasive micropapillary, invasive cribriform, metaplastic, adenoid cystic, and apocrine carcinomas). Nottingham combined histologic grades were available only for cancers present in excision or mastectomy specimens because, at our institution, we do not routinely grade carcinomas present in core needle biopsy specimens. Among the 498 cases for which histologic grade was recorded, 116 (23.3%) were grade 1, 235 (47.2%) were grade 2, and 147 (29.5%) were grade 3.

The frequency distribution of ER immunohistochemical results based on the estimated percentage of ER+ tumor cells is given in Figure 1. In 157 cases (19.0%), the tumor cells showed complete absence of nuclear staining for ER, whereas in 660 cases (80.0%), 70% or more of the tumor cells were ER+. In the remaining 8 cases (1.0%), the proportion of ER+ tumor cells ranged from 20% to 60%. All 8 of these cases were in-house core needle biopsy specimens. Thus, in 817 cases (99.0%), the tumor cells were completely ER– or were strongly positive.

The frequency distribution of ER immunohistochemical results based on the Allred score is shown in Figure 2. Again, in 157 cases (19.0%), the tumor cells showed complete absence of nuclear staining for ER and were assigned an Allred score of 0. In 661 cases (80.1%), the Allred score was 7 or 8, indicating relatively strong and diffuse ER positivity. Thus, among the 825 cases in this analysis, the Allred score was 0 or was 7 or 8 in 818 cases (99.2%). Among the other 7...
cases, the Allred score was 5 in 3 cases and 6 in 4. All 7 of these cases were in-house core needle biopsy specimens.

**Discussion**

The results of this analysis of more than 800 ER immunostains performed on primary invasive breast cancers from a 2-year period indicate that with the anti-ER antibody and the method used for ER immunohistochemical analysis in our laboratory, the distribution of ER staining results was bimodal and that cases with weak ER staining are encountered only rarely. In particular, in more than 99% of cases we studied, the tumors were completely ER− or unequivocally ER+. There were only a few cases in this series, accounting for approximately 1% of the study population, in which the proportion of ER+ cells was other than 0 or more than 70% or in which the Allred score was something other than 0 or 7 or 8. The results of the present study are similar to those reported by Nadji et al. In that study, almost 6,000 breast cancers were evaluated for ER expression by immunohistochemical analysis with the same anti-ER antibody used in the present study (clone 1D5). These authors found, as we did, that most tumors were uniformly ER+ or completely ER−. In particular, 92.0% of the ER+ tumors showed diffuse, intense immunoreactivity. ER staining was more variable in 8.0% of their ER+ cases, but in most of those cases, this was attributable to inadequate fixation or tumor necrosis.

Until fairly recently, ligand-binding assays, such as the dextran-coated charcoal assay, were the standard methods used to determine the ER status of breast cancers, and the results of such assays were reported in a quantitative manner (as fmol/mg of protein). Studies of ER status using these assays revealed a broad range of values among ER+ breast cancers. As a consequence of this experience with biochemical assays, there had been (and still remains) the expectation that immunohistochemical assays for ER should result in a similar distribution of results, with a similarly broad range of values among ER+ cases. In fact, numerous studies have advocated the use of computer-assisted image analysis to quantify the ER content of breast cancers so that results of immunohistochemical assays could be reported in a manner analogous to that of the ligand-binding assays. However, this approach presupposes that there is a direct, linear relationship between the amount of ER present in the tumor cells and the amount of ER antigen detected by immunohistochemical analysis. While some studies certainly have suggested such a direct relationship, the association between the actual quantity of ER protein in the tumor cell nuclei and the apparent amount of ER antigen demonstrated by immunohistochemical assays is highly complex and may be as much a function of preanalytic factors (such as details of tissue fixation and processing) and assay sensitivity as of the actual amount of antigen present in the tumor cells.

A number of recent studies that support this contention are particularly noteworthy. Rhodes et al, based on data obtained from 66 laboratories participating in a United Kingdom external quality assurance program, found that ER immunohistochemical staining results were highly affected by the efficiency of the antigen-retrieval step and that this was, in fact, the single most important factor contributing to interlaboratory reproducibility.

Goldstein et al noted that immunohistochemical staining results for ER were highly dependent on the time of tissue fixation. With the assay used in their laboratory, the minimum fixation time for optimal ER immunohistochemical staining results was 6 to 8 hours, regardless of specimen type or size. Of note in that study, underfixation had a more detrimental effect on immunohistochemical staining results than did overfixation.

Vassallo et al performed immunohistochemical assays for ER on 20 invasive ductal carcinomas using 2 anti-ER antibodies
(1D5 and 6F11), 2 antigen-retrieval methods, and 3 detection systems. Thus, for each case, 12 different technical variations were studied. ER immunohistochemical results were scored semiquantitatively on a scale of 0 to 4. In 5 of 20 cases, the ER scores varied from 0 to 4, and in 3 cases, the scores ranged from 1 to 4, depending on the assay conditions used. Moreover, there was not a single case in which all 12 assay variations resulted in the same immunohistochemical staining score.18

Umemura et al17 studied 44 breast cancers with a biochemical assay and with 2 immunohistochemical assays, one considered by the authors to be “highly sensitive” and the other “non–highly sensitive.” The same anti-ER antibody (1D5) was used in both immunohistochemical procedures. These authors found that the non–highly sensitive procedure resulted in a broad distribution of ER staining and that the correlation with the biochemical assay was linear. In contrast, the highly sensitive procedure resulted in an increase in the proportion of positive tumor cells and the staining intensity compared with the non–highly sensitive procedure. This resulted in a shift of cases toward the higher end of ER positivity, and this, in turn, resulted in a nonlinear correlation with the biochemical assay.17

Taken together, the results of these studies highlight the critical role of preanalytic factors and assay details in determining the distribution of ER immunohistochemical results in any given population and, in our view, make it difficult to justify the routine use of quantifying ER immunohistochemical results in clinical practice.

It could be argued that the bimodal distribution of ER staining we observed in our cases is the result of using an immunohistochemical assay that is too sensitive and perhaps not sufficiently specific. However, given that we have not encountered, nor have we seen reported, false-positive nuclear staining with the ER antibody used in our laboratory (clone 1D5), we believe that it is more important to use an assay that is unlikely to produce false-negative results than to be concerned about shifting weakly or moderately positive results toward the higher end of ER positivity. This view is supported by the study by Harvey et al.5 which showed that the presence of as few as 1% weakly staining tumor cells in formalin-fixed, paraffin-embedded tissue samples using anti-ER antibody clone 6F11 is sufficient to predict a significant benefit from endocrine therapy. Although it might not be possible to generalize these results to ER immunohistochemical protocols that use other primary antibodies or different methods, they do suggest that the presence of virtually any ER signal in formalin-fixed, paraffin sections of breast cancers seems to be clinically important in predicting hormonal responsiveness, and this, in turn, would argue in favor of the use of a highly sensitive assay. Similar observations have been made by Barnes et al.3

Furthermore, in current clinical practice, once a case is considered to be ER+, the degree of ER positivity has no impact on recommendations for the use of hormonal therapy.19 Thus, ER is viewed by clinicians as a dichotomous rather than a continuous variable when assessing patient suitability for antiestrogen therapy.19 With this in mind, the most important consideration in the use of an ER immunohistochemical assay should be the correct identification of tumors with even low levels of ER expression as being ER+; the shifting of cases with low or intermediate levels of ER positivity to higher levels of positivity is of no clinical consequence.

Although the results of this study demonstrate that weakly ER+ tumors are rare using the method employed in our laboratory, other studies have clearly shown that there is considerable interlaboratory variability in the identification of tumors with lower levels of ER expression.60 It could, therefore, be argued that it might be difficult to generalize the results of our study to other institutions. However, there is no a priori reason to believe that results similar to ours would not be attainable elsewhere. The ER immunohistochemical method used in our laboratory is straightforward and uses commercially available supplies and reagents. Our results, we believe, are a function of careful attention to technical details of the assay, the use of appropriate control samples (including the requirement for the presence of ER+ normal ductal-lobular epithelium to consider a tumor ER– with certainty), a high index of suspicion when unexpected results are encountered (eg, an ER– tubular carcinoma), and participation in internal and external quality control and quality assurance programs.

The results of this study have implications regarding the appropriate threshold for ER positivity that should be used in clinical practice when ER is assessed by immunohistochemical analysis, an issue that remains a matter of considerable debate. While any institution that performs ER immunohistochemical analysis ideally should calibrate its assay to clinical outcome,5 this is not feasible at many (if not most) institutions because to do so would require an analysis of ER results in relation to outcome in a large cohort of patients treated with hormonal therapy. Instead, many institutions have adopted an arbitrary threshold for ER positivity, such as more than 5%, more than 10%, or even more than 20% ER+ tumor cells.2,5 Our data suggest that with currently available reagents and antigen-retrieval methods, the issue of what constitutes an appropriate threshold for ER positivity is moot because the ER immunohistochemical results will be essentially binary (completely negative or unequivocally positive). In our practice, there are very few cases in which the issue of threshold for positivity even arises (ie, cases with weak ER expression). Rather than using an arbitrary cutoff to categorize such cases as positive or negative, we report them as “low positive,” but we believe that such cases should be considered ER+ for treatment purposes, based on the data.
The only cases we report as ER– are those in which there is complete absence of tumor cell staining for ER.

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References