Original Article

ER-negative /PR-positive Breast Carcinomas or Technical Artifacts in Immunohistochemistry?

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Abstract

Background: Evaluation of estrogen (ER) and progesterone (PR) receptors is important in the management and prognosis of breast cancer patients. Immunohistochemistry (IHC) is currently the worldwide accepted methodology for detection of ER/PR receptors in breast carcinomas. However, technical artifacts may alter the results. Since most authorities believe that there are no true ER-negative/PR-positive breast tumors, therefore we hypothesized that technical artifact in IHC might cause ER-negative/PR positive cases.

Methods: The clinical records of 2432 patients treated by surgery at six community hospitals for different histologic subtypes of breast carcinoma were reviewed. Among them, 43 (1.8%) patients reported as ER-negative/PR-positive were re-evaluated in a reference laboratory. Expressions of ER and PR were evaluated by IHC on the same paraffin block used for the initial testing.

Results: The repeat study showed that of the 43 patients with the initial results of ER-negative/PR-positive, 24 (55.8%) were ER-positive/PR-positive, 15 (34.9%) were ER-negative/PR-negative, and 4 (9.3%) were ER-positive/PR-negative. In none of the 43 cases were the initial results (ER-negative/PR-positive) confirmed.

Conclusions: Technical artifacts in IHC may alter ER/PR results in breast carcinomas. The technical factors affecting steroid receptor IHC ought to be properly controlled to provide reliable results.

Keywords: Breast carcinoma, estrogen receptors, immunohistochemistry, progesterone receptors

Introduction

Assessment of steroid receptor status has become the standard of care for patients with breast cancer. Immunohistochemistry (IHC) is now the globally accepted methodology for detection of estrogen (ER) and progesterone (PR) receptors in breast carcinomas.[1] Both ER and PR show nuclear expression in positive cases. ER content, in particular, is correlated with prolonged disease-free survival and increased likelihood of response to hormonal therapy. The study of ER status by IHC analysis has been proven to have higher discriminating power than biochemical assays for predicting disease-free and overall survival.

PR expression is reported along with ER expression, and IHC determination of PR expression has now been clinically validated.[2] An accurate ER/PR IHC result is critical to initiate targeted therapy and endocrine therapy that are the standard of care in breast cancer to suit the unique biologic tumor characteristics in each individual patient. Patients with ER-positive/PR-positive tumors have a better prognosis than patients with ER-positive/PR-negative tumors, who in turn have a better prognosis than patients with ER-negative/PR-negative tumors.[3] Most authorities believe that there are no true ER-negative/PR-positive breast tumors. Since we have noticed a relatively large number of ER-negative/PR-positive breast

tumors, we re-evaluated these tumors for their ER and PR status by repeating ER and PR IHC according to standard techniques. The aim of study was to examine possible technical or analytical pitfalls leading to misinterpretation of ER/PR status.

Materials and Methods

The medical records of 2,432 female patients in whom post-lumpectomy or mastectomy for breast carcinoma were performed and who were initially diagnosed between October, 1992 and May, 2004 were retrospectively retrieved from six different community hospitals. Among these, 43 (1.8%) were diagnosed with breast carcinomas that were ER-negative/PR-positive. The clinical history, pathology report, and hematoxylin and eosin (H&E) slides from all 43 patients were reviewed. The H&E stained slides were submitted to an internationally accredited reference laboratory that was under the supervision of a pathologist who was an expert in IHC. The original ER/PR stains were not available for review by the pathologists.

H&E slides of the cases were reviewed by two pathologists and a representative block of tumor was selected for each case. The corresponding paraffin embedded tissue block was recut in 3 μm sections and three unstained slides were prepared for each case. One slide was stained with H&E in a routine fashion.
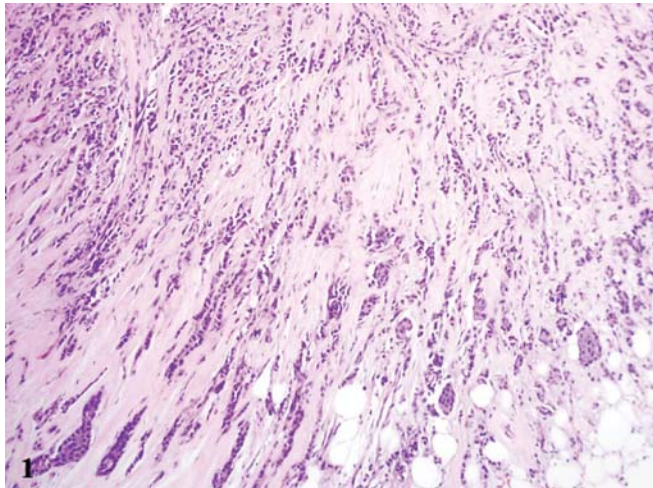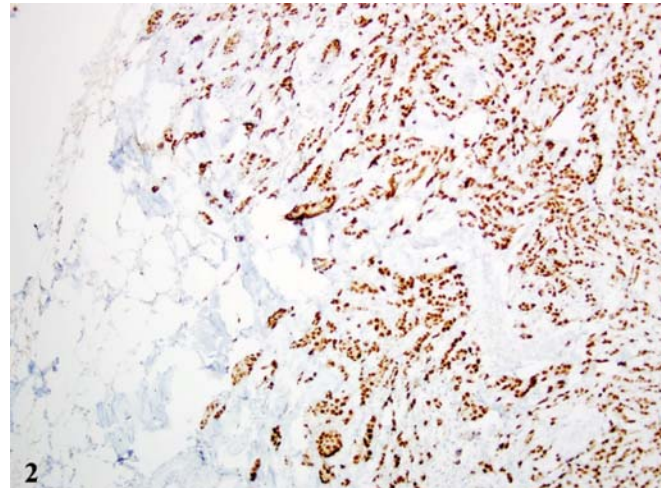
The IHC assays for ER and PR were performed on 3 μm section unstained slides from the paraffin blocks and float-mounted on plus-coated glass slides. The methodology for ER and PR was the same. For each antibody and each batch, positive and negative controls were used. Human endocervix was used as a positive control because of its easy availability and relatively stable reactivity. The negative control consisted of non-immune mouse IgG substituted for the primary antibody. Controls were run with each batch

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Accepted for publication: 14 December 2011

Table 1. Patients and types of carcinoma.

Carcinoma type	Patients (*n*)
Infiltrating ductal carcinoma*	26
Infiltrating and in-situ ductal carcinoma**	12
In-situ ductal carcinoma	4
Invasive lobular carcinoma	4
*Infiltrating ductal carcinoma included 23 conventional ductal carcinomas, 1 colloid carcinoma, 1 medullary carcinoma, and 1 papillary variant.	
**Infiltrating and *in-situ* ductal carcinoma included 11 conventional ductal carcinomas and 1 apocrine variant.	

Figure 1. Infiltrating ductal carcinoma (H&E stain 100×).

Figure 2. Strong nuclear expression of ER receptors (Immunohistochemical stain 100×).

of slides, at an average of 40 slides per batch. The essential steps of the IHC assay included blocking endogenous peroxide with a solution of 6% hydrogen peroxide for 3 minutes; antigen retrieval in a pressure cooker for 20 minutes at a temperature of 120ºC; blocking non-specific antigens by the Avidin/Biotin Blocking System (avidin solution for 10 minutes and biotin solution for another 10 minutes after rinsing off avidin); incubation with primary mouse monoclonal antibody for 30 minutes in a humidified chamber; linking with biotinylated (anti-mouse, anti-rabbit, and anti-goat) secondary antibodies for 25 minutes in a humidified chamber; and enzyme labeling with streptovidin peroxidase for 25 minutes. A biotinylated anti-mouse antibody was used at a 1:10 dilution for ER and an anti-mouse antibody (1:100 dilution) was used for PR. The repeat H&E stained (Figure 1) slides and repeat ER and PR stains were reviewed by two pathologists, independent from the primary reviewers. Nuclear staining of any intensity was considered positive in all PR and ER immunohistochemical staining cases (Figures 2 and 3).

Results

The patients' ages ranged from 27 to 67 years old (mean age: 46.6 years). All specimens were excisional biopsies. Histologic findings on H&E slides are summarized in Table 1. Tumor grading reported for 29 cases was: grade I (2), grade II (8), and grade III (19). There was no documented information regarding transportation time of fresh tissue to fixative, fixation type, duration of fixation, method of IHC staining, or IHC stain analysis and interpretation.

ER and PR analyses were semiquantitative. The results of the repeat IHC for ER and PR are summarized in Table 2. In none of the 43 cases was the initial result of ER-negative/PR-positive confirmed.
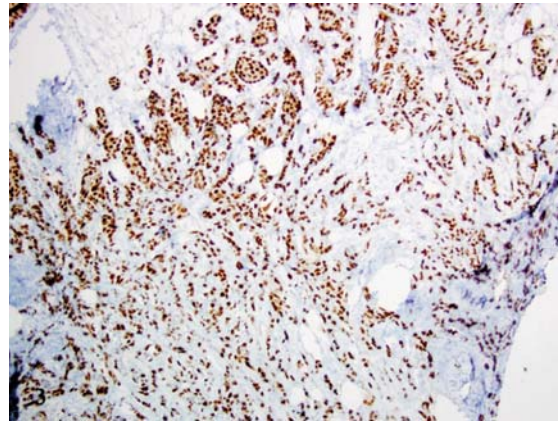
Discussion

IHC is the standard detection method for evaluating ER/PR expression levels in invasive breast carcinoma. Consistent IHC ER/PR results are important because they are integral in determining hormone therapy. The presence of ER, as detected by IHC, is a weak prognostic marker of clinical outcome in breast cancer[4] but a strong predictive marker for response[5] to tamoxifen-based therapy. Recent studies have demonstrated that ER expression is present in approximately 70% of breast cancers, [6] so an accurate and reliable ER result is critical for hormone therapy. ER status is strongly influenced by tumor grade and histology.[7] Nadji et al.[6] in a study of almost 6000 tumors, have noted that most grade I tumors are ER-positive, as are pure tubular, colloid, and classic lobular carcinomas. In our study, low grade tumors such as infiltrating lobular carcinoma,[1] and colloid carcinoma[1] have been initially reported as ER-negative/PR-positive.

PR expression is generally reported along with ER expression. It has further been suggested that PR status is independently associated with disease-free and overall survival, that is, patients with ER-positive/PR-positive tumors have a better prognosis than patients with ER-positive/PR-negative tumors, who in turn have a better prognosis than patients with ER-negative/PR-negative tumors.[3] PR analysis can provide important prognostic information and prediction of response to adjuvant hormone therapy in ER-positive tumors.[8]

As with all IHC studies of therapeutic targets, accurate and perhaps quantitative assessment of the results is critical. There are several major factors that can dramatically affect the apparent ER and PR status of a breast carcinoma as determined by IHC, including tissue fixation, choice of anti-ER or anti-PR antibody,

Table 2. The results of repeat ER/PR and their correlation with types of carcinomas.

Carcinoma type	ER-positive/PR- positive	ER-positive/PR-negative	ER-negative/PR-negative
Infiltrating ductal carcinoma*	16	1	9
Infiltrating and in-situ ductal carcinoma**	6	2	4
In-situ ductal carcinoma	1	1	2
Invasive lobular carcinoma	1	N/A	N/A
Total= 43	**24**	**4**	**15**
*Colloid ($n = 1$) and papillary ($n = 1$) type infiltrating ductal carcinomas were ER-positive/PR-positive. *Medullary ($n = 1$) variant of infiltrating ductal carcinoma was ER-negative/PR-negative. **Apocrine ($n = 1$) variant of infiltrating and in-situ ductal carcinoma was ER-positive/PR-positive.		

Figure 3. Strong nuclear expression of PR receptors (Immuno-histochemical stain 100×).

and determination of thresholds for reporting immunostaining and reproducibility.[9]

Breast resection specimens must be sectioned and placed in fixative as quickly as possible and the time recorded. Tissue sections must be immersed in an adequate volume of fixative (ratio of tissue/fixative= 1:20) within a maximum of one hour from surgery.[10] The pathologist (or pathology assistant) should cut a 2-mm thick sample of tumor, together with a 2-mm thick sample of benign breast tissue and place them both into the same cassette at the time of the initial evaluation, thus ensuring that normal breast elements are available as appropriate internal tissue controls for subsequent breast marker testing.[10] Approximately eight hours of fixation in 10% formalin is the minimum fixation time for consistent IHC ER results for both core and excisional biopsies. Less than eight hours may allow ER to be washed away during the dehydration steps of processing which may lead to spuriously low or negative ER/PR values.[11] The fixative should be a 10% aqueous phosphate-buffered, 4% formaldehyde [pH 7.0–7.4 (10% phosphate-buffered formalin)] for breast tissue samples. It is true that formalin will penetrate smaller samples more quickly than larger samples, but actual fixation is a chemical reaction that takes time.[12]

Breast cancer specimens should be processed in conventional processors.[10] The temperature of the tissue processor should not exceed 37°C, paraffin in tissue processors or embedding centers should not be warmed over 60°C, and the tissue should not be kept in paraffin for an extended time.[10,13] We had no records of fixation time or type for our samples.

Having a record of the fixation time for each breast tissue sample is expected to prove valuable for interpreting and troubleshooting aberrant and/or unexpected ER results. ER IHC assays that are negative in well-differentiated cancers such as a tubular carcinoma or classic lobular carcinomas are such examples. Positive and negative controls should be included with every ER IHC batch run.[10]

Although a number of anti-ER antibodies are available, the ideal antibody is one that is both robust and has been clinically validated. To date, there are only three such antibodies, 1D5[14], 6F11[1,15], and SP1[15] clones, which have all been demonstrated to produce results that correlate with clinical outcome; all have also been demonstrated to be equal or superior to ligand-binding assays in this respect.[1,14–16] Published data further suggest that the SP1 rabbit monoclonal may be the most robust of these reagents and better in identifying those patients most likely to respond to tamoxifen than the 1D5 clone.[15] Earlier studies from three decades ago had suggested that the ER-negative/PR-positive group of tumors corresponded to about 10% of all cases.[17] However, more recent studies using more robust antibodies have suggested that this latter group probably represents one composed of false-negative ER results; with optimal immunohistochemical methods, the number of tumors in this subset is near zero, or zero.[6]

In our study, ER was expressed in 28 cases and PR expressed in 24 cases. None of the cases were ER-negative/PR-positive. Collins et al. have reviewed the ER immunostains of 825 breast cancers demonstrating that the overwhelming majority of breast carcinomas are either completely ER-negative or ER-positive, and cases with weak ER immunostaining are rare.[18] The controversy regarding the interpretation of what constitutes a positive ER result by IHC has been resolved by a statement issued in the November 1–3, 2000 National Institute of Health (NIH) consensus Statement on Adjuvant Therapy for Breast Cancer, which states: "any positive nuclear ER immunostaining is considered to be a positive result and should be a definitive reason for instituting antiestrogen therapy for a patient".[19]

The National Comprehensive Cancer Network (NCCN) Task Force Report has stated the main overall conclusions regarding ER as follows: "ER is a strong predictor of response to endocrine therapy; ER status of all samples of invasive breast cancer or duc-

tal carcinoma in situ (DCIS) should be evaluated by IHC; IHC measurements of PR, although not as important clinically as ER, can provide useful information and should also be performed on all samples of invasive breast cancer or DCIS; IHC is the main testing strategy for evaluating ER and PR in breast cancer and priority should be given to improve the quality of IHC testing methodologies; all laboratories performing IHC assays of ER and PR should undertake formal validation studies to show both technical and clinical validation of the assay in use; and all laboratories performing IHC assays of hormone receptors in breast cancer should follow additional quality control and assurance measures as outlined in the upcoming guidelines from the American Society of Clinical Oncology and College of American Pathologists."[20] Therefore, pathologists who report ER/PR results should become familiar with the correct interpretation of ER/PR expression. In our study, it is not clear how the primary pathologists have interpreted ER/PR expression. Low grade carcinomas that are shown to be ER-positive/PR-positive in large studies are interpreted as ER-negative/PR-positive by primary pathologists. This favors the possible pitfalls in processing or interpretation. This study has clearly shown that ER is reported as a false negative in 28 out of 43 patients (65%) and PR is reported as a false positive in 19 out of 43 patients (44%). This is not a minor difference between the results of two laboratories with minimal or no impact on patient care. The difference between the results of the original laboratories and the accredited reference laboratory raises a warning for possible pitfalls in the pre-analytical, and/or analytical phases of the process. The impact of false negative ER results on patient treatment is tremendous and may simply leave the patients with fewer and more aggressive treatment options.

Technical issues in performing IHC can potentially change steroid receptor results, adversely affecting patient care. Awareness of this issue will guide laboratory directors to re-evaluate their validation studies that are currently in use, and will prompt pathologists to repeat ER/PR tests if the results do not correlate with histology, particularly in cases of low grade carcinomas. Finally, patients with breast cancer will benefit from accurate ER/PR results.

References

1. Harvey JM, Clark CM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol*. 1999. **17:** 1474 – 1481.
2. Mohsin SK, Weiss H, Havighurst T, Clark GM, Berardo M, Roanh le D, et al. Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. *Mod Pathol*. 2004. **17:** 1545 – 1554.
3. Bardou VJ, Aprino G, Elledge RM, Osborne CK, Clark GM. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol*. 2003; **21:** 1973 – 1979.
4. Hahnel R, Woodings T, Vivian AB. Prognostic value of estrogen receptors in primary breast cancer. *Cancer*. 1979; **44:** 671 – 675.
5. Allred DC, Harvey JM, Berardo M, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol*. 1998. **11:** 155 – 168.
6. Nadji M, Gomez-Fernandez C, Ganjei-Azar P, Morales AR. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. *Am J Clin Pathol*. 2005; **123:** 21 – 27.
7. Anderson WF, Chatterjee N, Ershler WB, Brawley OW. Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database. *Breast Cancer Res Treat*. 2002; **76:** 27 – 36.
8. Rakha EA, El-Sayed ME, Green AR, Paish EC, Powe DG, Gee J. Biologic and clinical characteristics of breast cancer with single hormone receptor positive phenotype. *J Clin Oncol*. 2007; **25:** 4772 – 4778.
9. Gown AM. Current issues in ER and HER2 testing by IHC in breast cancer. *Mod Pathol*. 2008; **21 (suppl 2):** S8 – S15.
10. Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, Hammond EH, Hewlett B, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. *Appl Immunohistochem Mol Morphol*. 2008; **16:** 513 – 520.
11. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. *Am J Clin Pathol*. 2003; **120:** 86 – 92.
12. Fox CH, Johnson FB, Whiting J, Roller PR, Formaldehyde fixation. *J Histochem Cytochem*. 1985; **33:** 845 – 853.
13. Taylor CR. Immunomicroscopy: a diagnostic tool for the surgical pathologist. *Major problems in pathology.* Philadelphia: Saunders; 1986: xviii, 452 p. (2 col. plates).
14. Barnes DM, Harris WH, Smith P, Millis RR, Rubens RD. Immunohistochemical determination of oestrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients. *Br J Cancer*. 1996; **74:** 1445 – 1451.
15. Cheang MC, Treaba DO, Speers CH, Olivotto IA, Bajdik CD, Chia SK, et al. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. *J Clin Oncol*. 2006; **24:** 5637 – 5644.
16. Pertschuk LP, Feldman JG, Kim YD, Braithwaite L, Schneider F, Braverman AS, et al. Estrogen receptor immunocytochemistry in paraffin embedded tissues with ER1D5 predicts breast cancer endocrine response more accurately than H222Sp gamma in frozen sections or cytosol-based ligand-binding assays. *Cancer*. 1996; **77:** 2514 – 2519.
17. Osborne CK, Yochmowitz MG, Knight WA 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer*. 1980; **46 (suppl 12):** 2884 – 2888.
18. Collins LC, Botero ML, Schnitt SJ. Bimodal frequency distribution of estrogen receptor immunohistochemical staining results in breast cancer: an analysis of 825 cases. *Am J Clin Pathol*. 2005; **123:** 16 – 20.
19. Eifel P, Axelson JA, Costa J, Crowley J, Curran WJ Jr., Deshler A, et al. National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1–3, 2000. *J Natl Cancer Inst*. 2001. **93:** 979 – 989.
20. Allred DC, Carlson RW, Berry DA, Burstein HJ, Edge SB, Goldstein LJ, et al. NCCN Task Force Report: Estrogen Receptor and Progesterone Receptor Testing in Breast Cancer by Immunohistochemistry. *J Natl Compr Canc Netw*. 2009; **7 (suppl 6):** S1 – S21; quiz S22 – S23.