Immunohistochemistry of Estrogen and Progesterone Receptors Reconsidered

Experience With 5,993 Breast Cancers

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Abstract

Paraffin sections or fine-needle aspiration smears from 5,993 cases of invasive mammary carcinomas were assessed immunohistochemically for estrogen receptor (ER; 1D5) and progesterone receptor (PR; 636) expression. Staining pattern and intensity were correlated with histologic subtypes and nuclear grades of tumors.

Positive nuclear staining for ER and PR was observed in 75% and 55% of invasive carcinomas, respectively. In 92% of ER+ cases, diffuse and uniform staining of most tumor cells was observed. In the remaining 8%, a focal ER reaction was seen, usually because of inadequate fixation. In 21% of PR+ tumors, the reaction was heterogeneous or focal but unrelated to fixation. There were no ER–, PR+ tumors. All pure tubular, colloid, and infiltrating lobular carcinomas were ER+. All medullary, apocrine, and metaplastic and most high-nuclear-grade carcinomas were ER–.

With monoclonal antibody 1D5 and antigen retrieval, immunohistochemical reaction for ER in breast cancer usually is an all-or-none phenomenon; therefore, quantitation of results is unnecessary. Despite antigen retrieval, inadequate fixation can cause false-negative results; evaluation of internal positive control samples is imperative. ER positivity and negativity are predictable in certain histologic types and nuclear grades of breast cancer. The reaction for PR can be heterogeneous or focal.

Analysis of steroid receptor status has become the standard of care for patients with breast cancer. Estrogen receptor (ER) content, in particular, has been correlated with prolonged disease-free survival and increased likelihood of response to endocrine therapy. Assessment of ER status by immunohistochemical analysis has been shown to have higher discriminating power than biochemical assays for predicting overall and disease-free survival.[1-4] Similarly, parallel studies have suggested that immunohistochemical detection of ER using the monoclonal antibody ER-1D5 and heat-induced antigen retrieval offers the highest sensitivity and predictive value for endocrine response.[2,5,6] There are, however, unresolved issues regarding tissue preparation, types of antibodies and detection reagents, and, most important, the methods of interpretation of results. Lack of technical standardization and interlaboratory reproducibility might influence the immunohistochemical assay's predictability of meaningful clinical end points.

For the past several years, immunohistochemical analysis for assessment of steroid receptors has been performed daily in our laboratory, on internal cases and on cases received from outside institutions. It became evident early on that with the use of ER-1D5 and heat-induced antigen retrieval, the reaction for ER in the tumor cells of the majority of breast cancers was uniformly positive or uniformly negative. The immunohistochemical reaction for progesterone receptor (PR) on the other hand, could be focal and quantitatively variable. Focal staining for ER was extremely unusual, and variation in staining intensity usually was due to inadequate fixation. Based on these initial observations, the concept of semiquantitation of the steroid receptor reaction was reevaluated. This study is based on the immunohistochemical staining of 5,993 breast cancer cases for ER and PR during a 6-year period.

Materials and Methods

Samples

During 6 years, 5,993 consecutive cases of breast cancer were stained and evaluated for ER. Of these, 5,497 were tissue samples that also were evaluated for PR. The remaining 496 specimens were fine-needle aspiration (FNA) cytology specimens in which only ER evaluation was performed.

Of the tissue specimens, 47% were from patients who were diagnosed and treated at our institution (University of Miami/Jackson Memorial Medical Center, Miami, FL); the remaining 53% represented referrals from other laboratories in South Florida and Latin America. The tissue fixative for all internal and 2,365 (81%) of the external cases was 10% phosphate-buffered formalin. We were not aware of the nature of the fixative in the remaining 555 (19%) referral cases. Fixation periods for our internal cases ranged from 6 to 18 hours.

H&E-stained slides of each case were reviewed, and the presence of invasive carcinoma was confirmed in all cases. The histologic type of each tumor was recorded. Only pure histologic subtypes (eg, pure tubular, colloid) were recorded as such. In a few cases in which the differential diagnosis between invasive ductal and lobular carcinoma was difficult with the routine stain, we used immunohistochemical analysis for E-cadherin (when the antibody became available). We did not encounter any cases of pleomorphic lobular carcinomas during this period. The nuclei of neoplastic cells from the cases of infiltrating ductal carcinoma, not otherwise specified were graded from 1 (small, uniform, evenly dispersed chromatin, micronucleoli, and rare mitoses) to 3 (large, highly pleomorphic with irregularly dispersed chromatin, macronucleoli, and frequent mitoses). The nuclear grades of lobular, medullary, and metaplastic carcinoma and of the special histologic subtypes of ductal carcinoma (eg, pure tubular, papillary, colloid) were not recorded.

During the same period, Papanicolaou-stained smears from 496 FNA specimens of infiltrating mammary carcinomas also were reviewed. All aspiration cytology slides were fixed in 95% isopropyl alcohol from 10 minutes to 18 hours and stained by using a standard Papanicolaou technique. Following microscopic examination, 1 representative diagnostic smear from each case was selected for ER immunohistochemical analysis. Immunohistochemical analysis for PR was not performed on cytologic samples. The nuclear grade of the aspirated tumor cells was recorded for each sample. For the purposes of the present study, the use of nuclear morphologic examination was preferred to the modified Nottingham score for assessment of grade because the former could be determined readily from the core needle biopsy specimens and aspiration cytology samples as reproducibly as from the excisional specimens.

Immunohistochemical Procedure

The step-by-step procedure is outlined in **Table 1**. Monoclonal antibody 1D5 (M7047, DakoCytomation, Carpinteria, CA) was used to detect ER. ER-1D5 is a mouse IgG antibody that reacts with the A/B region of the N terminal domain of ERα. For detection of PR, the monoclonal anti-PR antibody 636 (M3569, DakoCytomation) was used. For cytologic smears, the immunohistochemical procedure for ER was identical to the procedure used for the histologic slides and did not require destaining of the smears. Immunohistochemical analysis for PR was not performed on cytologic specimens.

Control Samples

Whenever possible, tumor blocks were selected that contained normal or nonneoplastic mammary epithelium to serve as positive internal control samples. The external positive control samples for ER and PR were cases of invasive mammary carcinomas. The antibody (negative) control sample consisted of replacement of the primary antibody with nonimmune mouse IgG on adjacent histologic sections.

Table 1
Stepwise Procedure for ER and PR Staining as Used in the Study*

1. Cut paraffin sections at 3.0 μm.
2. Melt paraffin by placing slides in a 58°C oven for 30 minutes or in a 37°C oven overnight; dewax in xylene.
3. Rehydrate slides in decreasing ethanol grades.
4. Block endogenous peroxidase by using a 6% solution of hydrogen peroxide in water (3.0 minutes, room temperature).
5. Place slides in target retrieval solution (S1699, DakoCytomation, Carpinteria, CA) and heat at 90°C in a vegetable steamer for 10 minutes.
6. Block endogenous biotin by using the biotin-blocking reagent (X0590, DakoCytomation).
7. Incubate with primary antibodies, ER-1D5 (dilution 1:25) and PR-636 (dilution 1:100), 22 minutes at room temperature (DakoCytomation).
8. Add the linking solution; biotinylated antimouse immunoglobulin; incubate for 22 minutes (K0690, DakoCytomation).
9. Add streptavidin-peroxidase conjugate and incubate for 22 minutes (K0690, DakoCytomation).
10. Place slides in diaminobenzidine solution for 10 minutes (K3468, DakoCytomation).
11. Apply 1% cupric sulfate (1.0 minute, room temperature) to intensify signal; counterstain with 0.2% fast green (2.0 seconds).
12. Dehydrate in increasing grades of ethanol, clear in xylene, and mount.

ER, estrogen receptor; PR, progesterone receptor.
* All washes and dilutions were made with tris-buffered saline (DakoCytomation, S1968). Steps 6 through 10 were carried out in an automated instrument (Autostainer Plus, DakoCytomation).

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Evaluation of Staining Results

Immunohistochemically stained slides were evaluated for the presence of positive reaction, cellular localization (nuclear or cytoplasmic), pattern of staining (focal or diffuse), and intensity of reaction in individual tumor cells (strong or weak). Any positive nuclear reaction for ER and PR, irrespective of percentage of reactive cells, was recorded as positive. In other words, there was no arbitrary percentage cutoff point used in this study. The intensity of positive nuclear reactions was evaluated against the reaction in respective internal control samples (whenever available) or the known positive external control sample.

Results

Frequency of ER and PR Positivity

The frequency of steroid receptor positivity in mammary carcinoma is summarized in **Table 2**, **Table 3**, and **Table 4**. Of all mammary carcinomas, 75% were positive for ER and 55% of all tumors reacted positively for PR. All PR+ tumors were positive for ER. In other words, there were no ER–, PR+ carcinomas in this series. Table 3 illustrates the relationship between receptor positivity and histologic subtypes of mammary carcinomas. All pure tubular, colloid, and invasive lobular carcinomas were positive for ER, whereas none of the classic medullary, apocrine, and metaplastic carcinomas contained the receptor. The reaction for PR was less predictable than ER among the pure tubular, pure colloid, and invasive lobular carcinomas. However, among the ER– subtypes, none were positive for PR. Among infiltrating ductal carcinomas of no special type, all nuclear grade 1 tumors contained ER, whereas only 2.0% of nuclear grade 3 carcinomas showed ER positivity.

Characteristics of ER Staining

The overall pattern of staining for ER and PR on histologic sections is depicted in **Table 5**. A positive reaction for ER was observed as brown-black, fine, intranuclear granules. The staining reaction in the normal ductal and lobular epithelium was heterogeneous, ie, strongly positive, weakly positive, and negative nuclei were seen side by side or grouped together **Image 1**. In contrast, the staining reaction for ER in most invasive mammary carcinomas that stained positively for this receptor was diffuse and uniform. In positive cases, the intranuclear staining reaction for ER was observed in more than 90% of the tumor cells throughout the lesion (Image 1). The overall intensity of the reaction in a given tumor usually was equal to or greater than that of the adjacent nonneoplastic epithelial cells whenever present **Image 2**. Variation in the intensity of the positive reaction among tumor cells was minimal. This phenomenon was best illustrated in well-fixed core biopsy specimens and in FNA specimens.

A focal positive reaction for ER was observed in 328 tumors (8%). This staining pattern was observed only in tissue sections from excisional biopsy specimens and, in the majority of cases, could be attributed to inadequate fixation or focal tumor necrosis. Artifactual focal staining owing to inadequate

Table 2
Status of ER and PR in 5,497 Cases of Infiltrating Mammary Carcinoma in Histologic Specimens

Receptor	No. (%)
ER+	4,100 (75)
PR+	3,016 (55)
ER+/PR+	3,016 (55)
ER+/PR–	1,084 (20)
ER–/PR–	1,397 (25)
ER–/PR+	0 (0)

ER, estrogen receptor; PR, progesterone receptor; +, positive; –, negative.

Table 3
Relationship of ER and PR to Histologic Subtypes of Mammary Carcinoma*

Type of Carcinoma	ER+	PR+
Infiltrating ductal, not otherwise specified (n = 4,396)	3,255 (74)	2,330 (53)
Tubular (n = 237)	237 (100)	225 (95)
Colloid (n = 184)	184 (100)	133 (72)
Papillary (n = 44)	44 (100)	35 (80)
Apocrine (n = 40)	0 (0)	0 (0)
Medullary (n = 96)	0 (0)	0 (0)
Metaplastic (n = 120)	0 (0)	0 (0)
Infiltrating lobular (n = 380)	380 (100)	293 (77)

ER, estrogen receptor; PR, progesterone receptor; +, positive.
* Data are given as number (percentage).

Table 4
Relationship of Estrogen Receptor to Nuclear Grade of 4,892 Ductal Carcinomas, Not Otherwise Specified*

Nuclear Grade	Estrogen Receptor–Positive
1 (n = 1,151)	1,151 (100)
2 (n = 3,298)	2,471 (75)
3 (n = 443)	9 (2)
Total (n = 4,892)	3,631 (74)

* Data are given as number (percentage).

Table 5
Pattern of ER and PR Staining*

Pattern	ER+ (n = 4,100)	PR+ (n = 3,016)
Diffuse	3,772 (92)	2,383 (79)
Focal	328 (8)	633 (21)

ER, estrogen receptor; PR, progesterone receptor; +, positive.
* Data are given as number (percentage).

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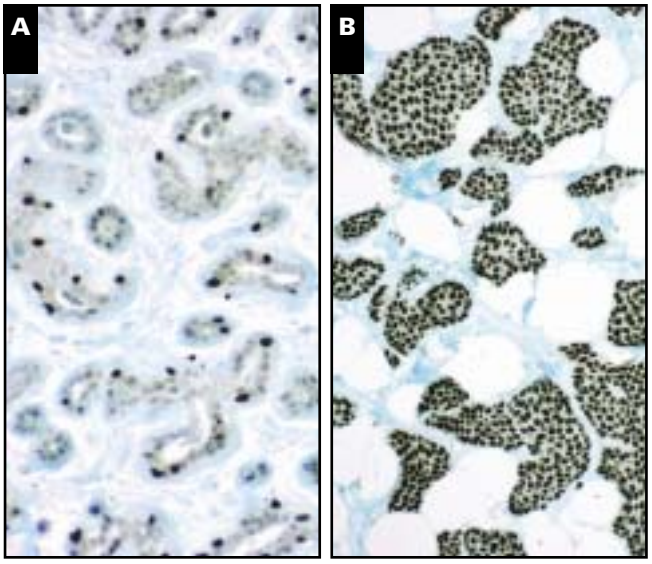

Image 1 **A**, Normal breast lobule. Heterogeneous estrogen receptor (ER) reaction; strongly positive, weakly positive, and negative nuclei are seen side by side (×100). **B**, Infiltrating mammary carcinoma, low nuclear grade. Uniform positive reaction for ER (×50).

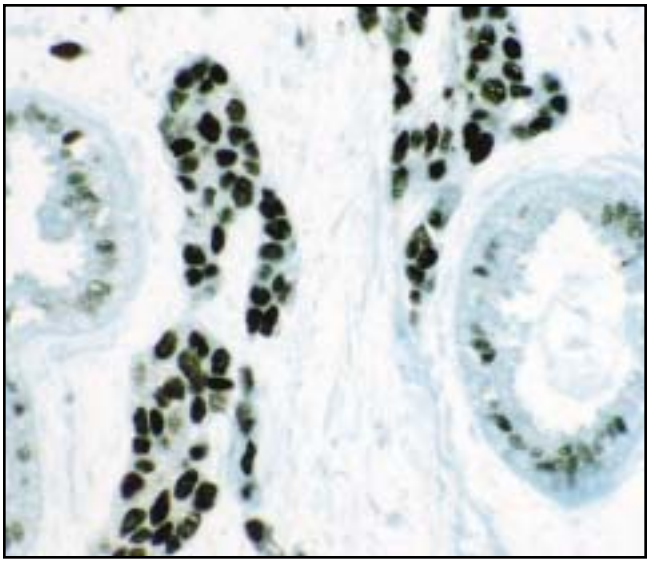

Image 2 Infiltrating ductal carcinoma, intermediate nuclear grade. Uniform positive reaction for estrogen receptor in tumor nuclei contrasts with the heterogeneous staining pattern of nonneoplastic ductal epithelium (×200).

fixation was characterized by a gradual decrease in the intensity of the reaction from the better-fixed periphery of the tissue section toward the center **Image 3**. Likewise, normal ductal and lobular epithelium in inadequately fixed areas of tissue did not stain for ER. In necrotic areas, loss of ER antigenicity preceded morphologic evidence of cellular necrosis. These areas, nevertheless, exhibited the same gradual loss of reaction intensity toward the center of necrotic focus **Image 4**. True focal staining for ER was exceedingly uncommon. In these rare cases, the areas of tumor that were negative for ER usually were sharply separated from the positively stained areas, appeared adequately fixed, and generally presented a different histologic appearance, eg, larger cells and pleomorphic nuclei. Whenever possible, negative reactions for ER in tumor cells were validated against the positive staining reaction of the internal control samples **Image 5**.

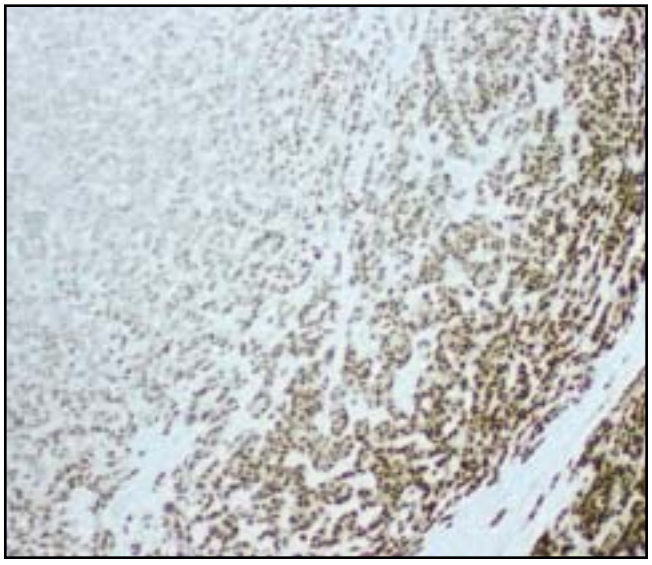

Image 3 Effect of inadequate tissue fixation on estrogen receptor staining. Note gradual loss of intensity from the better fixed periphery of the tissue toward the center (×50).

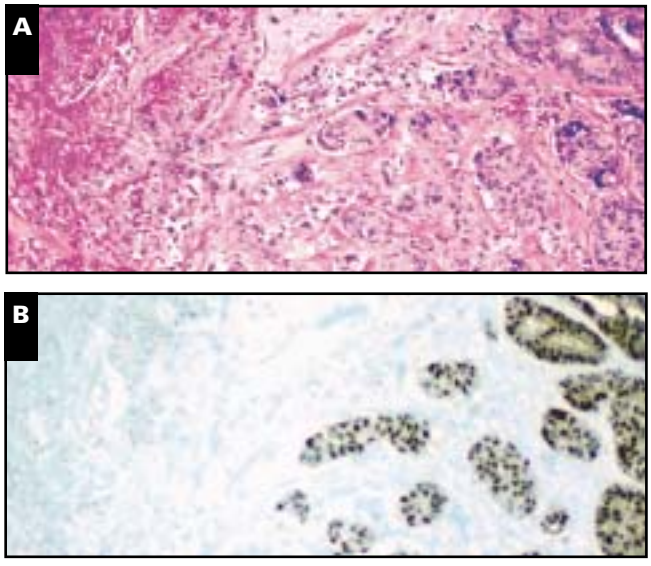

Image 4 Effect of tumor necrosis on estrogen receptor (ER) staining. Note the loss of reactivity for ER in necrotic tumor (**A**, H&E, ×50; **B**, ER, ×50).

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Characteristics of PR Staining

The staining of nonneoplastic epithelium for PR resembled that of ER, as it showed variation in pattern and intensity. Focal or heterogeneous positive staining for PR was seen in 21% of the carcinomas, including those diagnosed on core biopsy specimens. Contrary to what we observed for ER, the distribution of the negative areas did not suggest a fixation artifact because positive and negative tumor cells were seen side by side individually or in groups **Image 6**. It appeared that the epitope recognized by the anti-PR antibody used in this study tolerates variability in tissue fixation considerably better than the one recognized by the ER-1D5 antibody. On the other hand, and similar to ER, the reactivity for PR was lost owing to tumor necrosis.

Discussion

The advent of immunohistochemical analysis and heat-induced antigen retrieval has changed or modified several old concepts about localization of steroid receptors in breast cancer. Today, most laboratories exclusively use immunohistochemical analysis for evaluation of the ER and PR contents of mammary carcinomas. Despite the assay's popularity for steroid receptor evaluation, several technical and analytic aspects of the method remain to be clarified. One major analytic factor that has been shown to affect interlaboratory reproducibility is quantitation, or semiquantitation, of staining results.

Identification of an analyte in histologic sections is dependent on a multitude of preanalytic factors related to tissue handling from the time of surgical excision to immunohistochemical staining. These variables include the nature and consistency of the excised tissue, the interval between excision and fixation, type of fixative, duration of fixation, thickness of the tissue block submitted for processing, type and duration of the processing method, and the time lapse between microtomy and use of the section for immunohistochemical analysis. These important preanalytic factors that could affect greatly the result of immunostaining, however, have yet to be standardized. For this reason, pathologists have always been aware of the unreliability of quantitative immunohistochemical analysis for various antigens in archival fixed and processed tissue samples. But the stand-alone nature of steroid receptor testing and the adoption of quantitative reporting from the old biochemical methods of measurements have created an artificial "need" to produce numeric results for ER immunohistochemical analysis.

During 6 years, 5,993 consecutive cases of breast cancer were stained and evaluated for ER. Of these, 5,497 were tissue biopsy specimens that also were evaluated for PR. The remaining 496 specimens were FNA cytology specimens. With the use of the monoclonal anti-ER antibody 1D5 and the method detailed in Table 1, most breast cancers showed relatively homogeneous staining for ER, whereas the expression of PR was heterogeneous and focal in more than 20% of the cases. Variations in the staining pattern for ER occurred but in most cases could be attributed to factors related to tissue fixation and antigen preservation. The remainder of this

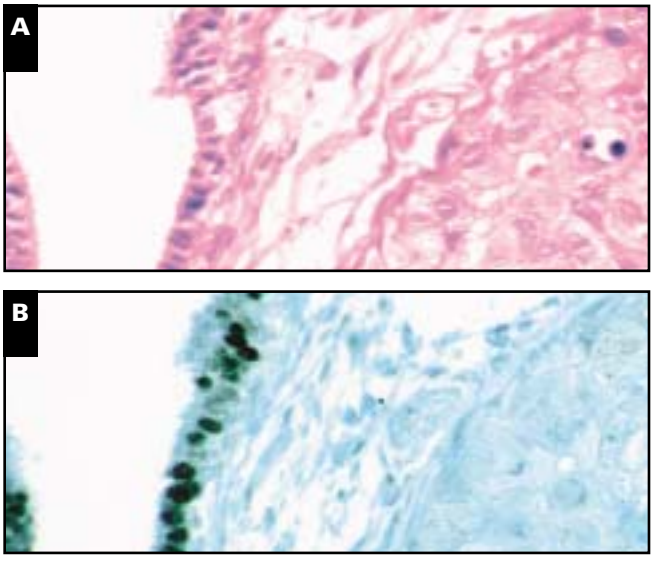

Image 5 **A**, Infiltrating ductal carcinoma, high nuclear grade (H&E, ×200). **B**, The negative estrogen receptor reaction in tumor cells is validated by the positive reaction in adjacent nonneoplastic ductal epithelium (×200).

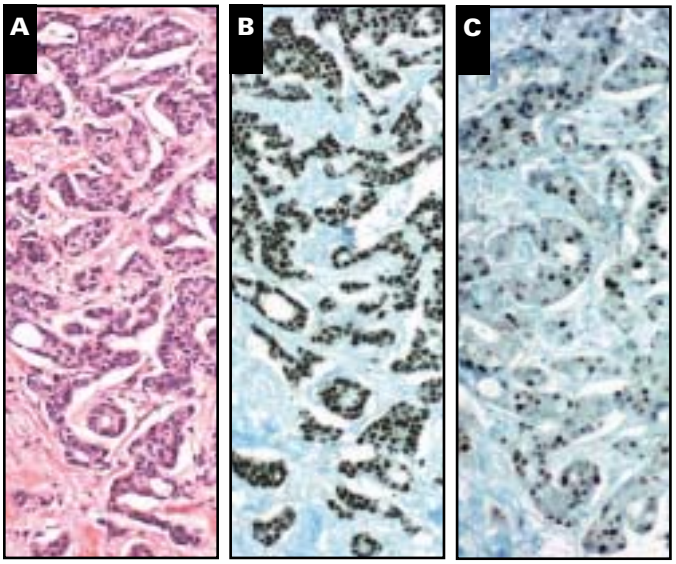

Image 6 Infiltrating ductal carcinoma. Uniform positive reaction for estrogen receptor (**B**) and heterogeneous staining reaction for progesterone receptor (**C**) (**A**, H&E, ×50; **B**, ×50; **C**, ×50).

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discussion outlines observations related to technical and analytic aspects of steroid receptor immunohistochemical analysis that might affect the results directly.

Internal positive control samples are extremely important for evaluation of steroid receptor immunohistochemical analysis; hence, every attempt should be made to select a block that contains normal or nonneoplastic elements. The optimal fixation time with 10% buffered formalin for tissue samples should fall within a range of 6 to 18 hours. It should be noted that this fixation period is recommended for adequately trimmed blocks (approximately 2.0 mm thick) and not for the whole lumpectomy or mastectomy specimen. In general, with prolonged fixation (more than a few days), there is an exponential decrease in the sensitivity of the immunohistochemical stains.[7] Heat-induced antigen retrieval can help reduce this problem, but it is ineffective in restoring the antigen that is lost owing to inadequate fixation, ie, lack of complete penetration of fixative into the center of the specimen. False-negative results might occur in unfixed areas, particularly when there is fibrosclerotic tumor stroma. This observation is supported by a recent report by Goldstein and colleagues,[8] who showed that even with antigen retrieval, the fixation time for reproducible ER staining in well-trimmed breast cancer tissue should be at least 6 to 8 hours.

Most mammary carcinomas are diffusely positive or completely negative for ER-1D5. One may argue that this observation could be the result of an inordinately high sensitivity of the immunohistochemical system in this study. This however, is not the case because normal or nonneoplastic mammary epithelium in the same histologic section adjacent to carcinoma had a heterogeneous staining pattern for ER-1D5, with negative, weakly positive, and strongly positive nuclei arranged side by side. Furthermore, when the same staining system is used for gynecologic tumors, positive results frequently are focal and have variable intensity (data not shown). Finally, well-fixed samples, including core needle biopsy specimens and aspiration cytology specimens, rarely show focal staining for ER-1D5. A representative sampling problem, therefore, is not an issue with 1D5 as documented by previous observations showing complete concordance of ER staining between fine- and core-needle biopsy samples and their corresponding excision specimens.[9,10]

Uniform expression of ER and most other biomarkers in breast cancer reflects current concepts about the biology of these neoplasms, ie, human mammary carcinomas are monoclonal in origin.[11] This is in contrast with other common cancers such as prostatic carcinomas in which the great majority are polyclonal and phenotypically heterogeneous.[12] The quantitative variability seen with PR, on the other hand, might be a reflection of functional variability of ER in some breast cancers.

It should be noted that not all immunohistochemical antibodies for ER produce a uniform staining result in breast cancer. The all-or-none staining results obtained with ER-1D5 might be explained by the fact that this antibody recognizes the A/B region of the N terminus of the ERα. This epitope typically is present in breast cancers with hormone-dependent, as well as hormone-independent, functional ER splice variants.[13,14] Focal ER staining may be seen with other antibodies used for immunohistochemical analysis such as 6F11. These antibodies are against the full-length of the ERα molecule and might not recognize ER splice variants that could be present in varying proportions of the tumor cell population.

Recently, Fisher et al found that in patients with node-positive breast cancer, the any-or-none ER results were not only simple and practical to report, but also were clinically relevant in predicting overall survival (E.R. Fisher, MD, verbal communication, February 2004). Our study demonstrates that quantifying ER immunoreactivity is not necessary and, hence, has no practical value. A simple report of the ER result as positive or negative provides the most useful information for the treating clinician. Quantitation of PR results is a matter to consider, although our experience has shown that clinicians have no use for such information.

ER results also are predictable in certain histologic types of breast cancer, and, in the absence of such correlation, one should suspect a technical problem. Pure tubular, colloid, and classic lobular carcinomas are almost always positive, whereas medullary, metaplastic, and apocrine carcinomas are consistently negative. In ductal carcinomas of no special type, the antigen identified by ER-1D5 is present in practically all of nuclear grade 1 and almost 75% of nuclear grade 2 tumors. Nuclear grade 3 carcinomas usually are negative for ER-1D5. The latter observation might be related to our higher threshold of atypicality required for a grade 3. Unlike ER, the positivity for PR could not be predicted from the histologic types. On the other hand, all ER– subtypes (medullary, metaplastic, and apocrine) also were negative for PR.

All PR+ cases in our study also were ER+. When using ER-1D5, we have yet to encounter a breast cancer that is negative for ER and positive for PR. Such negative ER results have been reported with assays that require an intact hormone-binding domain in the ER receptor such as ligand binding assays and some immunohistochemical methods.[1,15] This is not observed when the ER-1D5 antibody is used, possibly because it is able to recognize ER splice variants that might be missing the hormone-binding domain but contain the hormone-independent transcriptional function located at the A/B region of the N terminus.[13,16]

In adequately fixed specimens when ER-1D5 antibody is used in conjunction with antigen retrieval, most breast cancers are diffusely positive or completely negative for ER. This makes quantitation of ER results unnecessary. On the other hand, because the expression of PR could be focal, one may consider quantitating the results. However, the clinical value

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of PR staining in general and its quantitation in particular remains questionable. Most aberrant focal ER staining is due to technical factors, usually inadequate fixation. Therefore, the evaluation of internal control samples, ie, normal and nonneoplastic glands, is critical. The reaction for ER also correlates closely with certain histologic types and nuclear grades of mammary carcinomas. Finally, ER–, PR+ breast cancers are not encountered when ER-1D5 antibody is used.

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