

Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer

Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred

Purpose: Immunohistochemistry (IHC) is a newer technique for assessing the estrogen receptor (ER) status of breast cancers, with the potential to overcome many of the shortcomings associated with the traditional ligand-binding assay (LBA). The purpose of this study was to evaluate the ability of ER status determination by IHC, compared with LBA, to predict clinical outcome—especially response to adjuvant endocrine therapy—in a large number of patients with long-term clinical follow-up.

Patients and Methods: ER status was evaluated in 1,982 primary breast cancers by IHC on formalin-fixed paraffin-embedded tissue sections, using antibody 6F11 and standard methodology. Slides were scored on a scale representing the estimated proportion and intensity of positive-staining tumor cells (range, 0 to 8). Results were compared with ER values obtained by the LBA in the same tumors and to clinical outcome.

Results: An IHC score of greater than 2 (corresponding to as few as 1% to 10% weakly positive cells) was

used to define ER positivity on the basis of a univariate cut-point analysis of all possible scores and disease-free survival (DFS) in patients receiving any adjuvant endocrine therapy. Using this definition, 71% of all tumors were determined to be ER-positive by IHC, and the level of agreement with the LBA was 86%. In multivariate analyses of patients receiving adjuvant endocrine therapy alone, ER status determined by IHC was better than that determined by the LBA at predicting improved DFS (hazard ratios/ $P = 0.474/.0008$ and $0.707/.3214$, respectively) and equivalent at predicting overall survival ($0.379/.0001$ and $0.381/.0003$, respectively).

Conclusion: IHC is superior to the LBA for assessing ER status in primary breast cancer because it is easier, safer, and less expensive, and has an equivalent or better ability to predict response to adjuvant endocrine therapy.

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THE ESTROGEN RECEPTOR (ER) content of breast carcinomas is important as a prognostic and predictive biomarker, according to recently published guidelines,¹⁻³ and evaluation of ER status is part of the routine assessment of these neoplasms. Most of the data on the clinical utility of ER content have been generated using biochemical ligand-binding assays (LBAs), such as the dextran-coated charcoal assay (DCCA). Since the first report of its independent prognostic significance almost two decades ago,⁴ the assessment of ER status by DCCA has been validated repeatedly and is generally regarded as the standard by which other methods are assessed. There are, however, problems associated with LBAs for ERs. They are technically challenging and expensive; require radioactive reagents and relatively

large amounts of fresh-frozen tissue; and are insensitive and nonspecific in accounting for differences in the cellular composition of samples, such as those with a low tumor cellularity or contaminating benign cells that might be ER-positive.

The development of highly specific monoclonal antibodies⁵ and immunohistochemistry (IHC) techniques to localize ERs⁶ provided the potential to overcome most of the difficulties inherent to LBAs. Compared with LBAs, IHC is easier to perform, less expensive, safer, applicable to a wider variety of samples (eg, cytologic preparations, frozen tissue sections, fixed archival tissue sections, etc), and more sensitive and specific in the identification of rare ER-positive tumor cells or contaminating ER-positive benign epithelium under direct microscopic visualization.

The ultimate usefulness of ER status assessment by IHC, however, resides in its ability to predict clinical outcome, especially response to hormone therapy. Many studies have evaluated the clinical relevance of measuring ER status by IHC, and the large majority reported statistically significant relationships with clinical outcome.⁷ Nonetheless, there were limitations associated with these generally positive studies. For example, the majority evaluated patient populations of mixed clinical stage and treatment status, making it nearly impossible to separate the prognostic from the predictive implications of their results. In addition, these

From the Department of Pathology, University of Western Australia, Nedlands, Western Australia, Australia; and Division of Medical Oncology and Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX.

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Address reprint requests to D. Craig Allred, MD, University of Texas Health Science Center at San Antonio, Department of Pathology, San Antonio, TX 78284-7750; email allred@uthscsa.edu.

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studies used many different antibodies and detection systems with unequal sensitivities and specificities on tissue samples prepared in diverse ways. The most problematic aspect was the use of a wide variety of techniques for scoring and interpreting results with arbitrary rather than clinically calibrated definitions of ER positivity. Despite these largely unresolved issues, most laboratories today have already converted to assessing ER status almost exclusively by IHC on routine archival (ie, formalin-fixed paraffin-embedded) tissue samples.

The purpose of this study was to resolve some of these issues by developing an IHC assay for archival tissue, using inexpensive commercially available reagents and an easy, reproducible scoring system calibrated to clinical outcome. The prognostic and predictive usefulness of this IHC assay was evaluated and compared with a standard LBA in a large group of patients with primary breast cancer and long-term clinical follow-up.

PATIENTS AND METHODS

Patient Population

Tumor specimens from patients with primary breast cancer in the San Antonio Tumor Bank were included in this study. Patients were diagnosed between 1973 and 1993 and had their ER statuses evaluated by LBA at the time of diagnosis in our laboratory. Selection criteria included presentation with primary breast cancer, sufficient tumor tissue remaining after LBA for additional IHC assays, and long-term follow-up for disease recurrence and death. A total of 1,982 patients who satisfied these criteria were chosen: 997 with negative axillary lymph nodes and 985 with positive nodes. Surgical treatment included modified radical mastectomy in 91% of the patients and lumpectomy in 9%. Postoperative radiation was used in 21%. After surgery, 35% received no additional therapy. The remainder received systemic adjuvant therapy in a routine clinical setting; this therapy consisted of chemotherapy alone in 13%, endocrine therapy alone in 26%, and combined chemotherapy and endocrine therapy in 13%. The status of adjuvant therapy was unknown in 5%. Patients were observed for disease recurrence and death as previously described.⁸ A total of 620 patients (31%) had experienced disease recurrence, and 734 (37%) had died by the time of analysis. The median follow-up period for patients still alive at the time of analysis was 65 months (range, 1 to 214 months).

LBA for ER

Breast tumor specimens were frozen in liquid nitrogen immediately after excision and then sent to the Steroid Receptor Laboratory at the University of Texas Health Science Center at San Antonio. The tumor tissues were pulverized in liquid nitrogen, and cytosols were prepared for the LBA as previously described.⁹ From 1973 to 1984, ³H estradiol was used as the labeled ligand. Since 1985, the standard multipoint DCCA had been modified to incorporate ¹²⁵I-labeled estradiol and ³H-R5020 in a single assay, allowing for the simultaneous determination of both ER and progesterone receptor statuses. Tumors with an ER content of ≥ 3 fmol/mg protein (the limit of detection in this assay) were considered to be ER-positive, based on studies calibrated to clinical outcome.¹⁰⁻¹² The pulverized tissue that remained after the

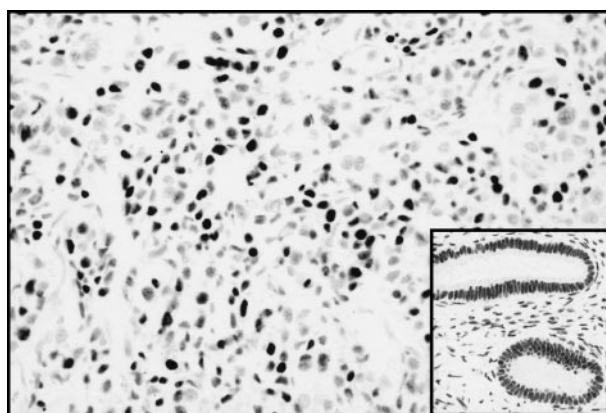


Fig 1. Photomicrograph of a representative invasive breast cancer tissue sample immunostained by the method used in this study (magnification, $\times 200$). ER-positive cells showed a dark brown or black nuclear signal. Using this field, this tumor would get a total immunohistochemical score of 6 (proportion score [= 4] + intensity score [= 2]). The inset shows human endocervix tissue, which was used as a positive control because of its easy availability and relatively stable reactivity.

corticosteroid receptor assay was performed was stored at -70°C for future use.

IHC for ER

Tissue sections for ER status determination by IHC were prepared from the pulverized frozen tumor specimens left over from the LBA as previously described.¹³ Because of the ultracold temperature used during pulverization, the tissue was fractured into histologically intact fragments ranging from approximately 0.1 to 1.0 mm in size. Individual samples consisted of 100-mg pellets of this particulate tissue, which was fixed for 6 to 8 hours in 10% neutral buffered formalin and routinely processed to paraffin blocks. Histologic sections from these samples resemble the large-core needle biopsies in routine clinical use today.¹³ The IHC assay was performed on 4 μm sections cut from the blocks and float-mounted on adhesive (silanized) glass slides. The essential techniques of the IHC assay included retrieving the antigen in 0.1 M boiling citrate buffer (pH 6.0) in a pressure cooker; blocking endogenous peroxidase with 0.1% sodium azide and 0.3% hydrogen peroxide; blocking nonspecific protein binding with 10% ovalbumin; binding with primary mouse monoclonal antibody 6F11 against the ER (Vector Laboratories, Burlingame, CA) at a dilution of 1:40 for 2 hours; linking with biotinylated rabbit antibody against mouse immunoglobulin G (Dako Corp, Carpinteria, CA) at a dilution of 1:100 for 30 minutes; enzyme labeling with streptavidin-horseradish peroxidase (Dako) at a dilution of 1:100 for 30 minutes; developing chromogen with 0.03% hydrogen peroxide and 1 mg/mL diaminobenzidine; enhancing the signal with 0.2% osmium tetroxide; and counterstaining with methyl green. Human endocervix tissue was used as a positive control because of its easy availability and relatively stable reactivity. The negative control consisted of nonimmune mouse immunoglobulin G substituted for the primary ER antibody. Controls were run with each batch of slides, at an average of approximately 50 slides per batch. The method produced a distinct nuclear signal in ER-positive tumor cells (Fig 1).

Immunostained slides were scored as previously described.^{7,14} In brief, each entire slide was evaluated by light microscopy. First, a proportion score was assigned, which represented the estimated propor-

tion of positive-staining tumor cells (0, none; 1, $< 1/100$; 2, $1/100$ to $1/10$; 3, $1/10$ to $1/5$; 4, $1/5$ to $2/5$; and 5, $> 2/5$). Next, an intensity score was assigned, which represented the average intensity of positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Slides were scored by pathologists who did not have knowledge of ligand-binding results or patient outcome.

Two pathologists (J.M.H. and D.C.A.) were trained and calibrated to use of the IHC scoring system by simultaneously evaluating a panel of 200 breast cancer tissue samples that were immunostained for ER and which were not part of the study presented here. They then independently scored another 220 cases that were part of this study. Their results (total scores) on the second panel of tumors were in complete agreement in 71% of the cases and within one IHC score in the remaining 29% of the cases. The weighted kappa statistic for concordance was 0.87 ($P < .0001$). Taken together, these results indicated that the scoring method was easy to learn and highly reproducible. Because the concordance between the pathologists was so high during the training, all further scoring of cases in this study was carried out by one pathologist (J.M.H.).

Statistical Methods

Associations between continuous variables were analyzed using nonparametric Spearman rank correlation coefficients. Associations between categorical variables were assessed by χ^2 tests. Kappa statistics were used as measures of agreement between the different pathologists and between the two methods for determining ER status. An optimal cut point for defining ER positivity was determined by computing log-rank statistics for each of the seven possible cut points of the total IHC score. Adjustments were made to the resulting P values, as suggested by Hilsenbeck and Clark.¹⁵ Univariate disease-free survival (DFS) and overall survival (OS) curves were estimated by the method of Kaplan and Meier and compared, using log-rank statistics. Cox proportional hazards regression models were created to assess the prognostic and predictive value of ER status in multivariate analyses. To adjust estimates of hazard ratios and their corresponding P values from Cox models for the multiple significance testing used to define the ER cut point, the following approach was used. The P value obtained from the Cox model was multiplied by seven (the number of possible cut points of the total IHC score). The Z statistic corresponding to this P value was obtained by inverting the cumulative normal distribution function. An adjusted parameter estimate for ER status was computed as the product of the Z statistic and the reported SE of the parameter estimate, based on the assumption that the bias associated with multiple significance testing primarily affects the magnitude of the parameter estimate rather than its SE. The adjusted hazard ratio and 95% confidence limits were obtained by exponentiation of the adjusted parameter estimate and its 95% confidence limits. Because all potential cut points are not biologically plausible and because this Bonferroni-type adjustment is known to be conservative, this technique probably overadjusts for the multiple significance testing used to define the IHC ER cut point. All analyses were performed using SAS (Version 6.11; SAS Institute, Cary, NC) on a Sun SparcServer 1000 system (Sun Microsystems, Inc, Mountain View, CA).

RESULTS

Agreement Between IHC and LBA for ER

A comparison of the distribution of IHC scores and ligand-binding values for ER status in the 1,982 tumors in this study is listed in Table 1. A nuclear signal for ERs, as

Table 1. Comparison of ER Status Results, as Determined by IHC and LBA in 1,982 Primary Breast Cancer Cases

IHC Score	Patients		Ligand Binding Results (fmol/mg protein)				
	No.	%	Mean	SD	Median	Minimum	Maximum
0	517	26	10	49	1	0	758
2	67	3	50	100	8	0	548
3	117	6	59	95	23	0	623
4	190	10	67	73	39	0	428
5	320	16	104	139	56	0	1549
6	370	19	141	158	89	0	1181
7	318	16	193	215	142	0	1798
8	83	4	282	312	185	0	1439

assessed by IHC, was observed in 74% of the tumors, with positive scores ranging from 2 to 8. The mean and median ligand-binding values for the same group of tumors increased monotonically as the IHC score increased, although there was considerable variability among tumors with the same IHC score. The Spearman rank correlation coefficient between the two techniques was 0.68 ($P < .0001$).

Defining ER Positivity by IHC

To identify a clinically meaningful cut point for defining ER-positive tumors, we examined DFS curves for all possible IHC scores within the different treatment groups. For patients receiving no systemic adjuvant therapy ($n = 701$), ER status was only a weak prognostic factor, as expected. The log-rank P value for the best cut point (IHC score > 4) in untreated patients was only marginally significant ($P = .024$) and became nonsignificant ($P = .20$) after adjustment for multiple significance testing. For patients who received adjuvant chemotherapy alone ($n = 407$), no significant cut points were identified (all $P > .40$). However, for patients who received adjuvant endocrine therapy, either alone ($n = 517$) or in combination with chemotherapy ($n = 260$), ER status was a highly significant predictor of DFS. For these latter two groups combined ($n = 777$), the best cut point (IHC score > 2) was highly significant ($P < .0001$) and remained so ($P < .01$) after adjustment for multiple significance testing. On the basis of these results, tumors were defined as ER-positive if their total IHC score was greater than 2 and ER-negative if their score was 0 or 2. Note that a total score of 3, the lowest possible positive score, corresponds to as few as 1% to 10% weakly staining tumor cells. When this definition was applied to the 220 training cases that were independently scored by both study pathologists, only two cases (1%) showed a discrepancy (ie, positive versus negative) in ER status, and in both cases, the tumors received a score of 2 by one pathologist and a score of 3 by the other (Fig 2).

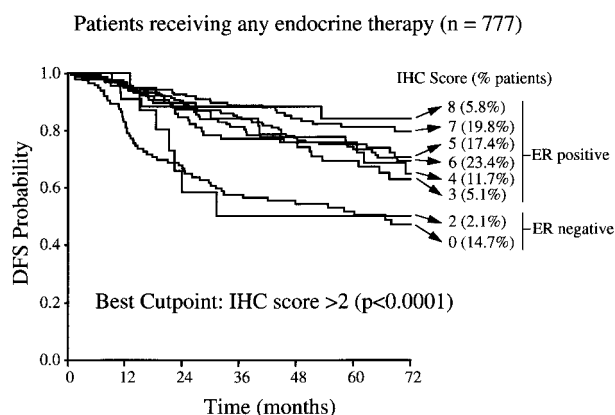


Fig 2. Univariate DFS curves for all possible total IHC scores in patients receiving any adjuvant endocrine therapy (almost always tamoxifen). An IHC score > 2 was the optimal cut point for predicting significantly improved outcome ($P < .0001$), and this value was used to define ER positivity throughout the study.

Using this definition of ER positivity, 70.5% of the 1,982 tumors in this study were ER-positive by IHC (ie, total score > 2), compared with 78.9% by LBA. Overall concordance between the tests was 85.5%. The kappa statistic for concordance was 0.62 ($P < .0001$). The remaining 14.5% of tumors had discordant results that fell into two groups. One group, comprising 11.4% of the tumors, was positive by LBA and negative by IHC. The LBA values were low (3 to 9 fmol/mg) in the majority of these tumors, but there was no overriding histologic explanation, such as the presence of ER-positive benign epithelium, to account for this discordant phenotype. The other group, comprising 3.1% of the tumors, was negative by LBA and positive by IHC. Again, there was no pervasive histologic feature, such as rare ER-positive tumor cells, that explained this discordant phenotype. When a cut point of 10 fmol/mg was used to define ER positivity by LBA, a standard used in many reference laboratories worldwide, the concordance between LBA and IHC assays increased slightly, to 87.7%.

Associations of ER by IHC With Other Standard Prognostic Factors

Table 2 shows the associations between ER status by IHC and other standard prognostic factors. Patients with positive axillary lymph nodes or with tumors larger than 2 cm in diameter had reduced frequencies of ER positivity ($P = .005$ and $P < .001$, respectively). ER positivity increased with advancing age, from 46% in patients younger than 35 years of age at diagnosis, to 65% in patients 35 to 65 years of age, to 82% in patients older than 65 ($P < .001$). Because this study was based on patients who had not been randomized to treatment, the rates of ER positivity differed with treatment status, as expected. For example, only 43% of patients who

received chemotherapy alone had ER-positive tumors, compared with 88% of patients who received endocrine therapy alone.

Clinical Utility of Assessing ER by IHC Versus LBA

The associations between ER status and clinical outcome were independently evaluated for IHC with unadjusted cut points; for IHC with adjusted cut points; for LBA using a cut point of 3 fmol/mg protein (LBA3, our clinically validated laboratory standard for 15 years); and for LBA using a cut point of 10 fmol/mg protein (LBA10, a common international laboratory standard) (Table 3). All analyses were adjusted for the contributions of standard prognostic factors (including axillary lymph node status, tumor size, and patient age at diagnosis) by Cox modeling for proportional hazards regression.

In the subset of patients receiving no adjuvant therapy (n = 688), ER positivity by LBA10 showed a marginally significant association with improved DFS, whereas IHC, adjusted IHC, and LBA3 were not significantly associated with DFS. Positivity results determined by IHC, LBA3, and LBA10 all showed significant associations with prolonged OS, whereas the association with adjusted IHC was marginal. Overall, the fractional hazard ratios for all statistically significant associations observed in this nonrandomized initially untreated group of patients were relatively large, emphasizing that ER status is a weak prognostic factor regardless of how it is measured, and were probably due in large part to responses to endocrine therapy given after first relapse in our study population.

In the subset of patients receiving adjuvant cytotoxic chemotherapy alone (n = 404), ER status by IHC, adjusted IHC, and LBA3 were not significantly related to DFS or OS.

Table 2. Relationships between ER Status Determined by IHC and Other Prognostic Factors

Factor	Patients		P
	No.	% ER-Positive	
Nodal status			.005
Node-negative	997	73	
Node-positive	985	68	
Tumor size, cm			< .001
≤ 2	667	78	
> 2	1294	67	
Patient age, years			< .001
< 35	81	46	
35-65	1181	65	
> 65	720	82	
Adjuvant therapy			< .001
None	701	72	
Chemotherapy alone	407	43	
Endocrine therapy alone	519	88	
Chemotherapy and endocrine therapy	261	73	

Table 3. Clinical Significance of ER Status Assessed by IHC; IHC Adjusted for Multiple Cut Points (IHC adj); LBA Using a Cut Point of 3 fmol/mg Protein (LBA3); and LBA Using a Cut Point of 10 fmol/mg Protein (LBA10)

Factor	No Adjuvant Therapy (688 patients)					
	Disease-Free Survival (220 relapses)			Overall Survival (263 deaths)		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
IHC	0.762	0.568-1.021	.069	0.685	0.518-0.906	.0079
IHC adj	0.900	0.672-1.207	.480	0.761	0.575-1.006	.056
LBA3	0.742	0.542-1.016	.062	0.793	0.586-1.075	.0001
LBA10	0.701	0.529-0.928	.013	0.679	0.519-0.887	.0046
Factor	Chemotherapy only (404 patients)					
	Disease-Free Survival (149 relapses)			Overall Survival (154 deaths)		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
IHC	1.008	0.734-1.383	.96	0.776	0.563-1.071	.12
IHC adj	1.008	0.734-1.383	.96	0.971	0.704-1.339	.86
LBA3	0.973	0.710-1.334	.86	0.823	0.597-1.134	.23
LBA10	0.748	0.536-1.043	.087	0.712	0.510-0.995	.047
Factor	Endocrine Therapy Only (517 patients)					
	Disease-Free Survival (130 relapses)			Overall Survival (159 deaths)		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
IHC	0.423	0.274-0.655	.0001	0.352	0.239-0.519	.0001
IHC adj	0.474	0.306-0.733	.0008	0.379	0.257-0.558	.0001
LBA3	0.707	0.356-1.404	.32	0.381	0.228-0.639	.0003
LBA10	0.699	0.426-1.145	.15	0.433	0.287-0.654	.0001
Factor	Chemotherapy and Endocrine Therapy (260 patients)					
	Disease-Free Survival (98 relapses)			Overall Survival (91 deaths)		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
IHC	0.491	0.320-0.753	.0011	0.502	0.315-0.801	.0038
IHC adj	0.559	0.365-0.858	.0078	0.590	0.370-0.944	.027
LBA3	0.513	0.307-0.856	.011	0.582	0.336-1.009	.050
LBA10	0.486	0.318-0.744	.0009	0.613	0.389-0.968	.036

NOTE. All analyses were adjusted for axillary lymph node status, tumor size, and age at diagnosis, by Cox modeling for proportional hazards regression.

LBA10 showed a marginally significant association with OS but was unrelated to DFS. Overall, the results in this nonrandomized group of high-risk patients initially treated with adjuvant chemotherapy also emphasize that ER status is a weak prognostic factor.

In clinical practice, ER status is used primarily as a predictive factor for response to adjuvant hormone therapy, rather than as a prognostic factor. In the subset of patients receiving adjuvant endocrine therapy alone (almost always tamoxifen; $n = 517$), ER positivity by IHC and adjusted IHC were both strongly associated with improved DFS (hazard ratios/ $P = 0.423/.0001$ and $0.474/.0008$, respectively) and OS (hazard ratios/ $P = 0.352/.0001$ and $0.379/.0001$, respectively). There were no significant associations between LBA3 or LBA10 and DFS, although ER positivity

by both assays was associated with improved OS (hazard ratios/ $P = 0.381/.0003$ and $0.433/.0001$, respectively). Overall, the results in this group of nonrandomized but similar patients emphasize that ER status is a strong factor for predicting response to adjuvant endocrine therapy and that IHC is somewhat better than LBA in this setting.

In the subset of patients receiving combined adjuvant chemotherapy and endocrine therapy ($n = 260$), ER positivity results determined by IHC, adjusted IHC, LBA3, and LBA10 all showed strong and essentially equivalent correlations with improved DFS and OS, showing again that ER is a strong predictive factor for response to endocrine therapy.

DISCUSSION

ER and progesterone receptor statuses measured by LBAs were the only prognostic and predictive biomarkers recommended for routine clinical use in breast cancer by the Tumor Marker Panel of the American Society of Clinical Oncology.³ In practice, their primary use is as predictive markers to distinguish patients who have little or no chance of benefiting from endocrine therapy from those who have some reasonable chance of benefiting. The justification for this endorsement was based on many studies conducted over the past two decades, involving patients in randomized clinical trials which showed that these tests were sufficiently sensitive, specific, and reproducible to reliably identify subsets of patients with significantly different risks for recurrence, survival, or treatment response.^{2,7,16} Nonetheless, many problems associated with LBAs have become increasingly urgent over the years, including high cost, technical difficulty, reliance on radioactive reagents, and, especially, a need for relatively large amounts of fresh-frozen tumor tissue. In addition, because they are based on whole-tissue homogenates, they are somewhat insensitive and nonspecific in accounting for differences in the cellular composition of samples, such as rare tumor cells or contaminating benign cells that might be ER-positive. These problems motivated research into alternative methods of assessing ER status, including IHC. IHC has several potential advantages over LBA, including lower cost, easier technology, greater safety, the ability to evaluate a wide variety of samples (eg, fine-needle aspirates, frozen tissue, fixed archival tissue, etc), and higher sensitivity and specificity in the identification of rare tumor cells or contaminating benign cells under direct microscopic visualization.

Since appropriate antibodies became available over 10 years ago, many studies have used IHC to evaluate ER status in breast cancers. Several studies compared ER status measured in the same tumors, using both IHC and LBA, and found 80% to 90% agreement between these tests.^{2,17} Many more studies, involving over 5,000 cumulative patients,

evaluated the relationship between ER status by IHC and clinical outcome in patients with breast cancer.¹⁸⁻³⁹ Nearly all of these studies showed a significant clinical benefit associated with the ER-positive phenotype, at least in univariate analyses and a few in multivariate analyses.^{30,34,35} However, most of these studies involved patient populations of mixed clinical stage and treatment status, making it difficult to separate the prognostic from the predictive implications of their results.

The few studies that specifically addressed subsets of patients not receiving any type of systemic adjuvant therapy^{19,27,33,35} found, on average, only approximately a 10% benefit in terms of DFS and/or OS associated with ER positivity as assessed by IHC, which is similar to results from earlier LBA studies and emphasizes that ER status is a very weak prognostic factor, regardless of how it is measured. The results of this study confirmed that ER status as determined by any method is a weak prognostic factor.

Several small studies have evaluated the ability of ER status determined by IHC to predict response to endocrine therapy in patients with advanced/metastatic breast cancer.^{21,28,40-57} In these studies, cumulatively involving over 1,000 patients treated with a variety of endocrine therapies, an average of approximately 70% with ER-positive tumors showed a significant clinical response, whereas approximately 85% with ER-negative tumors did not, which was a little better than results with ER statuses measured by LBAs in some of the same studies.⁷

Much less is known about the ability of ER status determined by IHC to predict clinical outcome in the far larger number of patients with less advanced disease who receive adjuvant endocrine therapy, which was the primary focus of this study. In our study, multivariate analysis of the subset of patients receiving adjuvant endocrine therapy alone (almost always tamoxifen; $n = 517$) revealed that ER positivity determined by IHC was superior to that determined by LBA at predicting prolonged DFS (hazard ratios/ $P = 0.423/.0001$ v $0.707/.03$, respectively) and roughly equivalent at predicting prolonged OS (hazard ratios/ $P = 0.352/.0001$ v $0.381/.0003$, respectively). Ferno et al,³⁶ in—to our knowledge—the only other similar study, also showed that ER positivity determined by IHC in archival tissue predicted significantly improved DFS in 98 patients receiving adjuvant tamoxifen therapy alone.

In the sense that nearly all studies to date have shown some clinical significance to assessing ER status by IHC, this methodology is approaching clinical validation, relative to published guidelines.¹⁻³ However, there are still persistent shortcomings in the technical validation of this test. For example, these studies used many different antibodies (eg, H222, H226, D547, D75, ID5) and a variety of usually

arbitrary methods for scoring and interpreting results. In addition, the majority utilized frozen-section IHC with antibody H222, which is very expensive and relatively insensitive in archival tissue (which has become the standard in most laboratories).

Our study developed and used an IHC assay for measuring ER status, based on inexpensive, highly specific, commercially available reagents that are sensitive in archival tissue. We also developed a method of scoring results that was easy to learn and highly reproducible. Most importantly, the definition of ER positivity was calibrated to clinical outcome, in that it was based on the IHC score identifying the largest number of patients with significantly improved DFS in response to adjuvant endocrine therapy, the primary reason in clinical practice for measuring ER status. With minimal training, pathologists in our laboratory were in agreement on discriminating positive from negative tumors in 99% of cases.

The optimal cut point in our study was a total IHC score of greater than 2, meaning that even patients whose tumors scored 3 (corresponding to as few as 1% to 10% weakly positive cells) had a significantly improved response, compared with those who had lower scores, and tumors with scores of 3 comprised 6% of our total study population. Our low cut point by IHC essentially agrees with previous studies using LBA, in which ER levels as low as 4 to 10 fmol/mg protein were associated with significant rates of response to endocrine therapy.¹⁰⁻¹² There may be several explanations as to why such low IHC scores predict favorable clinical outcome, including the possibility that the sensitivity of the test underestimates the proportion of ER-expressing cells or that low scores correspond to an ER-positive stem-cell population. Our IHC cut point also provided clinically significant results in various subsets of our study population (eg, OS in untreated patients, DFS and OS in patients receiving endocrine therapy alone, and DFS and OS in patients receiving combined endocrine therapy and chemotherapy), which partially satisfies the recommendation that the utility of prognostic/predictive factor assays be demonstrated in “test” and “validation” sets of patients.¹

Many hospital and commercial laboratories have converted to assessing ER status exclusively by IHC on archival tissue. They use diverse methodologies, and most have arbitrarily chosen 10% or even 20% positive tumor cells as their cutoff for defining ER positivity, potentially denying a substantial number of patients the benefits of adjuvant hormone therapy. Prudent laboratories offering ER status determination by IHC should perform rigorous validation studies themselves or follow the procedures of other laboratories that have.

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