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ESTROGEN RECEPTOR (ER) AND PROGESTERONE RECEPTOR (PgR), BY LIGAND-BINDING ASSAY COMPARED WITH ER, PgR AND pS2, BY IMMUNO-HISTOCHEMISTRY IN PREDICTING RESPONSE TO TAMOXIFEN IN METASTATIC BREAST CANCER: A SOUTHWEST ONCOLOGY GROUP STUDY

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Results of estrogen receptor (ER) and progesterone receptor (PgR) ligand-binding assays (LBAs) are strongly correlated with ER and PgR by immuno-histochemistry (IHC). To investigate whether ER and PgR by IHC are also strongly correlated with tamoxifen response, time to treatment failure (TTF) and overall survival (OS), the results of the 2 methods were directly compared in 205 patients with ER$^+$ metastatic breast cancer treated with daily tamoxifen (Southwest Oncology Group protocol 8228) with 9 years median follow-up. pS2, another estrogen-regulated molecule, was also analyzed. Tumors were scored for IHC from 0 to 5, according to the proportion of positively stained cells. These IHC scores for both ER and PgR were significantly associated with LBA levels ($p < 0.001$). There was a significant direct relationship between higher IHC ER, PgR and pS2 and increasing response to tamoxifen. TTF and OS were also significantly longer for patients with higher ER or PgR, but not pS2, IHC scores. Low, intermediate and high ER or PgR categories showed similar differences in response rates whether defined by LBA or IHC. In logistic regression models which included ER, PgR and pS2 by IHC; ER and PgR by LBA; and menopausal status, only ER (IHC) and pS2 (IHC) retained significance for predicting tamoxifen response ($p = 0.02$ and $p = 0.005$, respectively), along with menopausal status (for PgR by IHC, $p = 0.09$). Increasing ER and PgR by IHC, as by LBA, are thus significantly associated with a progressively better response and longer survival in ER$^+$ metastatic breast cancer. pS2 is also predictive in this setting. *Int. J. Cancer (Pred. Oncol.)* 89:111–117, 2000.
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Estrogen receptor (ER) status by ligand-binding assay (LBA) has long been used as a prognostic factor and a means to predict response to endocrine therapy. Depending on age and menopausal status, 50% to 80% of breast tumors are ER$^+$ by LBA (Thorpe *et al.*, 1987; Williams *et al.*, 1987; Nomura *et al.*, 1992), and response rate to first-line hormonal therapy for metastatic disease is about 50% to 60% (Bezwoda *et al.*, 1991). With the advent of monoclonal antibodies (MAbs) to ER protein, receptor status can also be evaluated by immunohistochemistry (IHC). Concordance between the 2 assays is high (80% to 90%) (Allred *et al.*, 1990; Stierer *et al.*, 1993; Molino *et al.*, 1997; Reiner *et al.*, 1986). Response rates have also been assessed using the IHC method, but studies for the most part have been small, uncontrolled and not prospective and have not had complete information for comparing ER measured by LBA and by IHC (Allred *et al.*, 1998; McClelland *et al.*, 1986; Manni *et al.*, 1980).

Progesterone receptor (PgR) and pS2 are ER-regulated proteins. The presence of PgR or pS2 should indicate a functional ER pathway, and indeed, the presence of PgR and pS2 in some studies was associated with improved prognosis and better response to endocrine therapy (Clark *et al.*, 1983; Gelbfish *et al.*, 1988; Stonelake *et al.*, 1994). There has been less published work comparing PgR by IHC to PgR by LBA, though correlation also appears high (Seymour *et al.*, 1990; Allred *et al.*, 1998; Pertschuk *et al.*, 1988; Gasparini *et al.*, 1992).

To directly compare receptor status measured by IHC and LBA and to determine if ER and PgR are as strongly correlated with clinical outcome and response to tamoxifen by IHC as by LBA, both methods were evaluated in a large group of patients with LBA-assessed ER$^+$ metastatic breast cancer with long-term follow-up. pS2, another estrogen-related molecule, was also analyzed by IHC.

MATERIAL AND METHODS

Eligibility

To be eligible for Southwest Oncology Group protocol 8228 (SWOG 8228) (Ravdin *et al.*, 1992), patients had to meet the following criteria: *(i)* metastatic breast cancer, *(ii)* ER level of >3 fmol/mg cytosolic protein in the primary or metastatic specimen, *(iii)* no prior treatment for metastatic disease, *(iv)* prior adjuvant chemotherapy or tamoxifen therapy allowed if completed more than 3 months prior to relapse, *(v)* PgR LBA performed, *(vi)* no massive liver involvement and *(vii)* signed an approved informed consent.

Patients and tumor specimens

SWOG 8228 was opened in 1982 and closed in 1987. There were 349 eligible patients. In the present ancillary study, SWOG 9314, formalin-fixed paraffin blocks from the primary or metastatic tumor were collected from 215 of these patients. For the remainder of the patients, blocks had been previously discarded or could not be located. Blocks from 5 patients could not be further analyzed because of poor fixation. Additionally, for 1 patient, the submitted specimen contained no invasive cancer. Four patients were not evaluable for response. Thus, a total of 205 patients were

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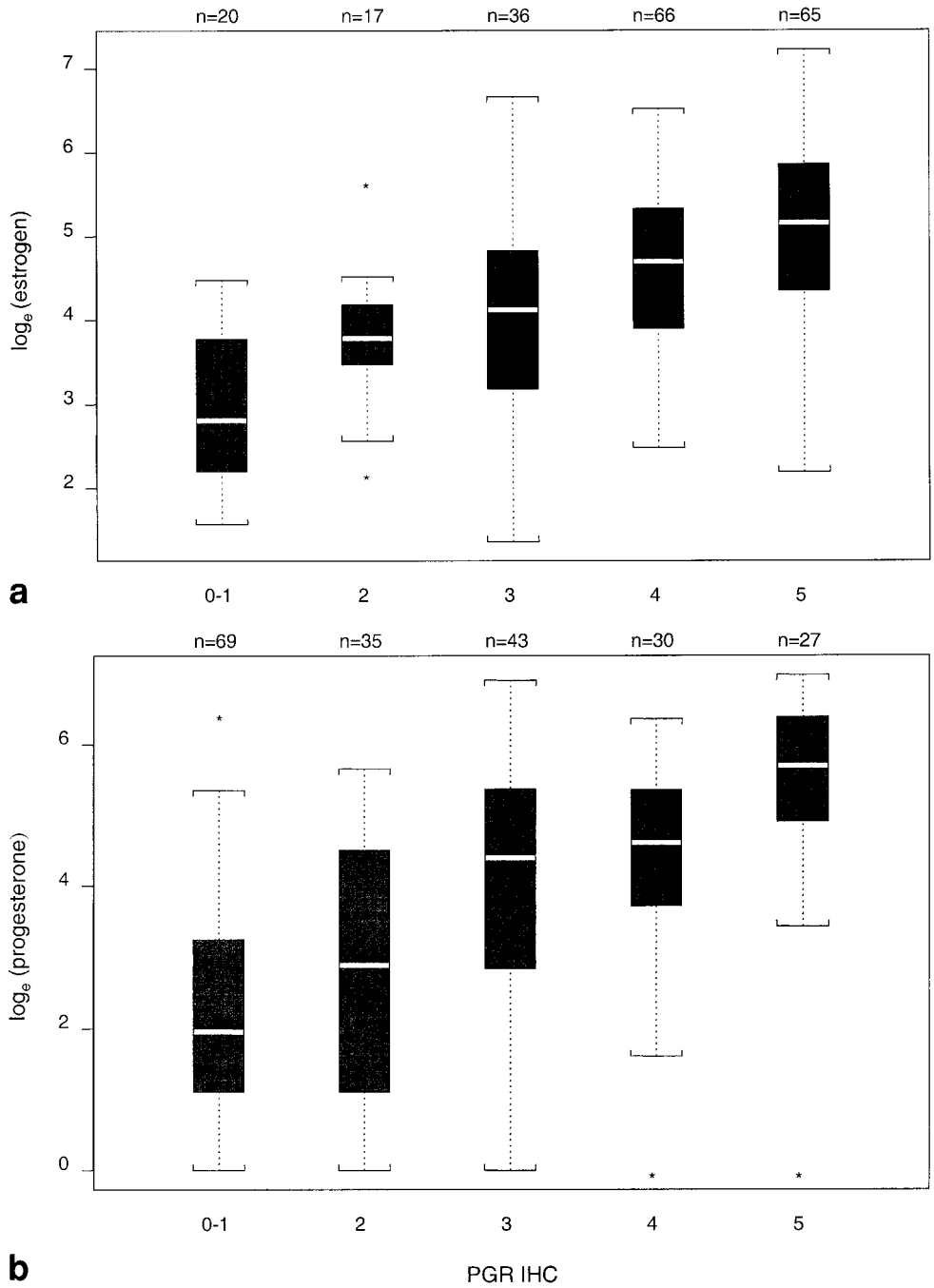

FIGURE 1 – Box plot presentation comparing receptor status measured by IHC or LBA. IHC scores of 0 and 1 were grouped together because of the low number of tumors with a score of 1. All values <1 were rounded to 1 so that all would have a log value of 0. Log is to the base e. The horizontal line in the mid-portion of the box represents the median. The upper and lower horizontal boundaries of the box represent the 75th and 25th percentiles, respectively. Stars represent outliers. The upper and lower brackets represent the largest and smallest values excluding outliers, respectively. *(a)* ER. *(b)* PgR. Results of the 2 methods were highly correlated.

analyzed. Patient and disease characteristics were similar to those in SWOG 8228, the participants of which were not registered to this study: PgR <10 fmol/mg, 29% *vs.* 32%; PgR >100 fmol/mg, 33% *vs.* 31%; ER <50 fmol/mg, 34% *vs.* 37%; pre-menopausal, 11% *vs.* 9%; age <65 years, 61% *vs.* 58%; visceral disease, 31% *vs.* 34%; no prior adjuvant therapy 78% *vs.* 77%; prior adjuvant tamoxifen, 3% *vs.* 4%. Only disease-free interval was statistically different for those in SWOG 9314, with fewer patients having a disease-free interval of >3 years, 19% *vs.* 26%, and a larger portion having metastatic disease at presentation, 47% *vs.* 34% (χ^2 2df, $p = 0.05$). The median follow-up of patients who remained alive was 9 years.

Analyzed tissue was from the following anatomic sites: 162 primary tumors, 15 skin/soft tissue, 18 lymph node, 4 lung, 5 bone, 1 ovary. Blocks were not categorized on the basis of histological type.

Treatment

In the initial phase of SWOG 8228, the first 87 patients were treated with tamoxifen 10 mg b.i.d. The dose was changed to 10

mg/m^2 b.i.d. for the remaining 255 patients. For SWOG 9314, 56 patients received tamoxifen 10 mg b.i.d. and 149 patients received 10 mg/m^2 b.i.d.

Response criteria

Complete response (CR) in patients without osseous involvement was defined as disappearance of all evidence of measurable or assessable disease for ≥4 weeks. In patients with osseous disease, CR was defined as the disappearance of all evidence of non-osseous cancer, bone scans or skeletal radiographs that showed no evidence of progression or new lesions, return of alkaline phosphatase to normal and disappearance of bone pain. In patients with only osseous disease, CR required complete normalization of radiographs and scans. Partial response (PR) in patients without osseous involvement was defined as a reduction of more than 50% in cross-sectional area of all measurable lesions for ≥4 weeks. In patients with osseous disease, PR was defined as a more than 50% reduction in cross-sectional area of all cancer in non-osseous sites, bone scans or skeletal radiographs that showed no evidence of progression or new lesions, reduction in alkaline phosphatase and improvement of bone pain. If only osseous disease was present, PR required a reduction in alkaline phosphatase with evidence of healing of lytic lesions and/or improvement in the bone scan. Stable disease was defined as a steady state or a response which was less than partial remission or progression. Progression was defined as the appearance of new lesions or an increase of more than 25% in the cross-sectional area of all measurable tumor over its minimal cross-sectional area or as a worsening of tumor-related symptoms in a patient with otherwise stable disease. Quality control of response evaluation was assured by the study and data coordinator's review of the submitted data.

Response to treatment was defined as CR, PR or prolonged stable disease (a time to treatment failure of >6 months). Prolonged stable disease was included as a response to treatment because patients with prolonged disease stabilization in response to tamoxifen clearly benefited clinically and because objective benefit is difficult to assess in patients with osseous disease. Time to treatment failure (TTF) was defined as the time from registration to first occurrence of progression, discontinuation of treatment or death. Physicians were informed that responses to tamoxifen might take 6 to 12 weeks to become clinically evident. The study design required tamoxifen to be continued for at least 4 weeks, even if there was initial progression or a tumor flare, and for at least 8 weeks in patients with stable disease.

IHC analysis

One 4 μm section of each submitted paraffin block was first stained with hematoxylin and eosin to verify that adequate numbers of invasive tumor cells were present and that fixation quality was sufficient for IHC analysis.

Serial sections (4 μm) were prepared from selected blocks and float-mounted on adhesive-coated glass slides (Superfrost/Plus Slides; Fisher, Pittsburgh, PA) for ER, PgR and pS2 immunostaining. The essentials of the immunoassay, in sequence, included quenching with 0.1% sodium azide/3% H_2O_2 for 30 min, blocking with 10% ovalbumin for 15 min, primary antibody overnight at room temperature, biotinylated rabbit anti-mouse (for ER and PgR) or swine anti-rabbit (for pS2) IgG linking antibody (Dako, Carpinteria, CA) at 1:100 for 30 min, streptavidin–horseradish peroxidase (Dako) at 1:100 for 30 min, hydrogen peroxide/diaminobenzidine chromogen, signal enhancement with 0.2% osmium tetroxide for 30 sec and methyl green counterstain. Antibody reagents were diluted in streptavidin-peroxidase diluent (Biogenex, San Ramon, CA). Autobuffer (0.1% BRIJ detergent in Tris buffer at pH 7.0) was used for intervening washes. Primary antibodies included 6F11 MAb (Novocastra, Burlingame, CA) at 1:40 dilution for ER, KD68 MAb (Abbott, Alameda, CA) at 5 μg/ml for PgR and polyclonal NCL-pS2 (Novocastra) at 1:250 dilution for pS2. Normal human endocervix and stomach were used as positive controls for ER and PgR and for pS2, respectively, because of their easy availability and high stable reactivity. Negative controls consisted of substituting non-immune mouse (for ER and PgR) or swine (for pS2) IgG at 5 μg/ml. Controls were included with each batch of approximately 50 slides. The method produced distinct nuclear (for ER and PgR) and cytoplasmic (for pS2) signals, as expected.

IHC scoring

Immunostained slides were scored as previously described (Allred *et al.*, 1998). Briefly, the entire slide was evaluated by light microscopy. A proportion score was assigned, representing the estimated proportion of positively staining tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; 5, >2/3). Any brown nuclear staining in invasive breast epithelium was counted toward the proportion score. There was no background nuclear staining in non-epithelial tissue. Slides were scored without knowledge of LBA results or patient outcome. Tumors with scores ≥2 were prospectively considered positive based on previous studies in our laboratory calibrating IHC scores to clinical outcome (Harvey *et al.*, 1999; Allred *et al.*, 1998).

Statistical analysis

χ^2 tests were used to test the association of ER and PgR by LBA with ER and PgR by IHC. TTF and overall survival (OS) were estimated using the Kaplan-Meier method. Log-rank statistics were used to compare TTF and survival. Multivariate analyses were performed using Cox's partially non-parametric model for censored survival data. The association of response with ER, PgR, pS2 and other characteristics was analyzed using logistic regression. All reported p values were 2-sided.

RESULTS

Correlation of ER and PgR results by LBA with those by IHC

Eligibility criteria required all tumors to be ER^+ by LBA. Ninety percent (185/205) of these tumors were ER^+ by IHC. There was a significant correlation between ER level measured by LBA compared with IHC ($p < 0.001$). Median ER level by LBA progressively increased with higher IHC scores (Fig. 1*a*). A similar pattern was also seen with PgR (Fig. 1*b*). Seventy-one percent (144/204) of tumors were PgR^+ by LBA (>10 fmol/mg) compared with 66% by IHC. Thirty-eight percent (23/60) of PgR^- tumors (<10 fmol/mg) were positive by IHC, while 22% (32/144) of PgR^+ tumors by LBA were negative by IHC. These findings are consistent with other published studies (Gasparini *et al.*, 1992; Stierer *et al.*, 1993; Reiner *et al.*, 1990).

Correlation of IHC ER with PgR and with response rate

There were significant direct relationships between higher IHC ER, PgR and pS2 and increasing response to tamoxifen (Table I). In logistic regression models, each of which included IHC ER, PgR and pS2 separately along with additional variables found to be important in SWOG 8228 [menopausal status, PgR (LBA) <10 fmol/mg and ER (LBA) <50 fmol/mg] (Ravdin *et al.*, 1992), IHC ER and pS2 were independently predictive of response ($p = 0.02$ and $p = 0.004$, respectively), along with menopausal status (for PgR by IHC, $p = 0.09$).

Correlation of IHC ER, PgR and pS2 with TTF and OS

Increasing levels of ER, PgR and pS2 by IHC were associated with longer TTF (Table II). Median TTF was approximately dou-

TABLE I – ER, PgR AND pS2 (IHC) *VS.* TAMOXIFEN RESPONSE (%)

Factor	IHC score			p value
	Negative (n)	Intermediate (n)	High (n)	
ER	25% (5/20)	46% (25/54)	66% (86/131)	0.001
PgR	46% (32/69)	55% (43/78)	70% (40/57)	0.03
pS2	52% (31/60)	48% (40/83)	72% (44/61)	0.01

ble for patients with high IHC ER or PgR (IHC score = 4 or 5) compared with those that were ER^- and PgR^- (IHC score = 0 or 1). Also, there was a progressive increase in OS as ER and PgR IHC scores became higher (Fig. 2*a*,*b*). Median survival for ER^- patients was only 17 months compared with 37 months for high ER patients. The same trend was also seen for PgR. Survival was not significantly different according to pS2 ($p = 0.86$) (data not shown).

TABLE II – ER, PgR AND pS2 (IHC) *VS.* TTF

Factor	IHC score (months)			*p* value
	Negative	Intermediate	High	
ER	5	4	10	0.003
PgR	5	7	10	0.007
pS2	7	5	11	0.08

In a multivariate analysis of TTF that included IHC ER, PgR and pS2 plus those factors which remained important in SWOG 8228 (LBA ER level, LBA PgR level, menopausal status and disease-free interval), IHC ER remained independently predictive ($p = 0.02$), while pS2 was marginal ($p = 0.06$) and PgR was not significant. In a multivariate model of OS which included IHC ER, PgR and pS2, as well as LBA ER and PgR status, disease-free interval, site of disease (visceral *vs.* non-visceral) and adjuvant therapy, IHC ER was marginally significant ($p = 0.06$), while PgR and pS2 were not.

Combining IHC ER with IHC PgR or pS2

Because both PgR and pS2 are ER-regulated proteins, their presence could reflect an intact ER pathway and, thus, a higher

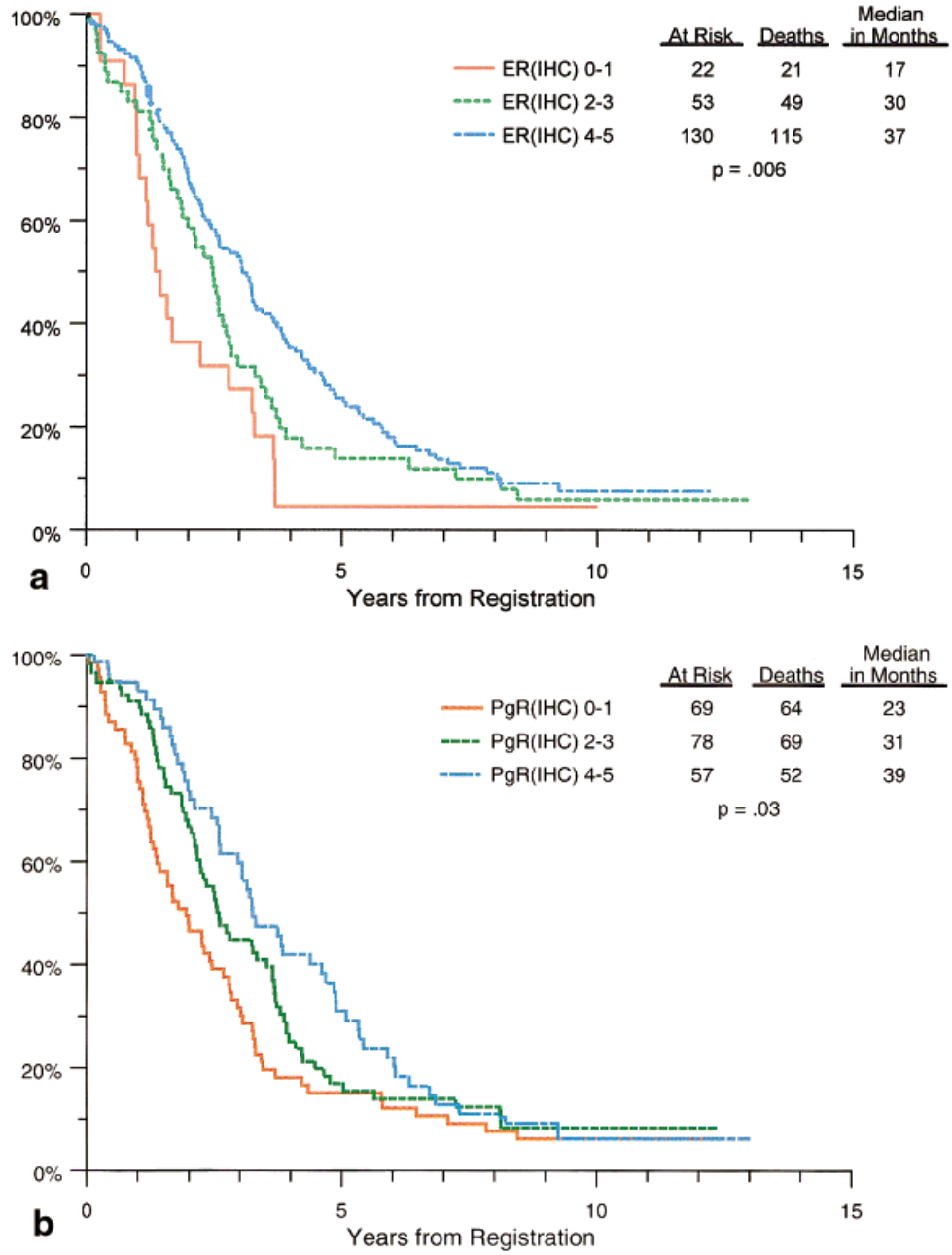

FIGURE 2 – Survival of patients according to IHC score. *(a)* ER. *(b)* PgR. Survival was progressively prolonged as the proportion of positively stained cells increased.

likelihood of response to tamoxifen. However, response, TTF or OS was not significantly improved for ER^+/PgR^+ patients over ER^+/PgR^- patients (Table III). pS2 status also was not additionally predictive (data not shown).

Comparative response rates for ER and PgR by IHC and LBA

The overall response rate for ER^+ tumors by IHC was 60% (111/185) *vs.* 56% (116/205) for those positive by LBA. For PgR^+ tumors by IHC, overall response was 61% *vs.* 59% by LBA. To determine if IHC might better predict response than LBA, results in low, intermediate and high categories were compared (Table IV). In general, tamoxifen response rates were similar for categories assessed by the 2 methods when comparing low/negative, intermediate and high categories. However, for lower ER LBA levels (<50), IHC did appear to add information. If IHC was negative, the response rate was only 25% but increased to 63% if IHC was high.

DISCUSSION

Measurement of ER and PgR in all invasive breast cancers is the standard of care in the United States (American Society of Clinical Oncology, 1996). ER and PgR are weakly prognostic but, more importantly, powerfully predictive of response to endocrine therapy. Receptor-positive tumors are at least 5-fold more likely to respond to endocrine therapy. LBA as a method to measure ER and PgR is a complex procedure that requires preserved frozen material, specialized equipment and radioactive pharmaceuticals. IHC, however, is simple and can be performed on routinely prepared, paraffin-embedded material without specialized equipment. These characteristics also allow it to be performed quickly on a large number of specimens. Thus, from a practical and logistical standpoint, IHC ER and PgR is clearly superior. If IHC were equal at predicting clinical outcome, it would be the preferred modality.

Our study examined the predictive value of ER and PgR using both types of assays. Only a small number of studies have compared the 2 directly with long-term assessment of clinical outcome (Hawkins *et al.,* 1988; Pertschuk *et al.,* 1996; De Lena *et al.,* 1988). This is the largest study of its kind that has been published, and the controlled, prospective design of the original clinical trial strengthens the validity of these findings. IHC ER and PgR were similar in their predictive value compared to LBA. The progressive relationship of increasing response rate as the ER and PgR score increases provides further evidence of quantitative value as a predictive marker. pS2, another estrogen-regulated protein, was also associated with improved response rate and TTF but not with survival.

There have been 21 studies assessing the ability of ER by IHC to predict response to hormonal therapy in patients with metastatic or advanced breast cancer (McCarty *et al.,* 1985; Pertschuk *et al.,* 1985, 1990, 1996; Ozello *et al.,* 1985; Jonat *et al.,* 1986; McClelland *et al.,* 1986, 1990; Berger *et al.,* 1987; Burton *et al.,* 1987; Coombes *et al.,* 1987; De Lena *et al.,* 1988; Andersen and Poulsen, 1988, 1989; Hawkins *et al.,* 1988; Gaskell *et al.,* 1989; Sklarew *et al.,* 1990; Nicholson *et al.,* 1991; Robertson *et al.,* 1992; Goulding *et al.,* 1995; Barnes *et al.,* 1996). In general, these were small studies that cumulatively involved only 1,291 patients. Our study is by far the largest on this issue and the only one based on a prospective clinical trial. In addition, the majority (15) of previous studies used antibody h222 on freshly frozen tumor samples. This is not very relevant today given that nearly all IHC for hormone receptors is performed on formalin-fixed, paraffin-embedded samples and that h222 is an expensive early-generation antibody that is not very sensitive on fixed tissue. We utilized an inexpensive, highly sensitive ER antibody (6F11) on formalin-fixed samples, which could be easily translated to routine clinical practice.

There have been only 3 previous studies assessing the predictive ability of PgR by IHC in 137 cumulative patients with metastatic or advanced breast cancer, with mixed results (Pertschuk *et al.,* 1988, 1990; Muller-Holzner *et al.,* 1993). All of these studies utilized freshly frozen tumor samples, which is not feasible in most clinical practices. Our results show a statistically significant relationship between PgR IHC phenotype and response to tamoxifen in over 200 patients, which makes an important contribution to this issue. We used a sensitive, commercially available antibody (KD68) on formalin-fixed samples, which could be easily translated to routine clinical practice.

Twenty patients, or 10%, were ER^- by IHC though ER^+ by LBA. Response rate in this group of patients was a surprising 25%. There could be a number of explanations. Because of tumor heterogeneity, sampling error could have occured. Different portions of the tumor may have been evaluated by each assay, and IHC may have evaluated an area composed only of a clonal expansion of ER^- tumor cells, while the remainder of the tumor was ER^+. Next, receptor status could have changed between primary and metastasis. Approximately 20% to 30% of ER primary tumors have ER^- metastases (Kuukasjarvi *et al.,* 1996). Of the 20 ER^- tumors, 15 were primaries and 5 were metastases. Thus, differences in receptor status of the primary and metastases could explain only a small part of this discrepancy. Observer variability in reading IHC slides, a semi-subjective procedure, could contribute to the difference. Indeed, when reviewed by a second observer, 2 of the 20 ER^- tumors were scored as low positive (IHC = 2). One was a long-term responder and the other, a non-responder. Lastly, it was observed that a number of the IHC ER^- tumors contained ER^+ non-invasive cancer or benign epithelium. By LBA, in which the whole tumor is homogenized, these cells could have produced a positive result.

Altogether, 5 of the 20 IHC ER^- tumors responded. Four of the 20 were PgR^+, and 2 of these 4 were among the responders. One

TABLE III – CLINICAL OUTCOMES OF IHC ER^+ PATIENTS ACCORDING TO PgR STATUS

Receptor status	Response % (n)	TTF (months)	OS (months)
ER^+/PgR^+	62 (81/131)	9	37[1]
ER^+/PgR^-	55 (29/53)	8	28[1]

[1] $p = 0.1$.

TABLE IV – IHC *VS.* LBA IN PREDICTING % TAMOXIFEN RESPONSE

	IHC score			Overall
	Negative (n)	Intermediate (n)	High (n)	
LBA ER				
<50	25% (4.16)	42% (11/26)	63% (17/27)	46% (32/69)
50–100	25% (1/4)	44% (7/16)	65% (15/23)	53% (23/43)
>100	— (0/0)	58% (7/12)	67% (54/81)	66% (61/93)
Overall	25% (5/20)	46% (25/54)	66% (86/131)	57% (116/205)
LBA PgR				
<10	51% (19/37)	40% (8/20)	— (3/3)	50% (30/60)
10–100	41% (12/29)	56% (18/32)	63% (10/16)	52% (40/77)
>100	33% (1/3)	65% (17/26)	71% (27/38)	67% (45/67)
Overall	46% (32/69)	55% (43/78)	70% (40/57)	56% (115/204)

of the responders was thought to be low ER^+ on re-evaluation. This leaves 2 tumors that were ER^-/PgR^- by IHC, even on review, and responded. Poor preservation and fixation of these older, archival specimens, resulting in false-negative results, might explain this, or perhaps there are pathways other than ER through which tamoxifen can produce a therapeutic response.

In summary, IHC ER and PgR have very similar prognostic and predictive value compared with LBA. Because IHC is easier and less expensive to perform, it would be a good alternative to LBA for evaluating hormone receptor status in breast cancer. Obtaining ER and PgR on all breast tumors should be the standard of care. Adequately fixed, well-preserved tumor tissue, along with a standardized staining process and a stringently low cut point (specimens with >1% of cells staining are considered positive) (Harvey *et al.*, 1999), is essential for obtaining accurate, reproducible, clinically useful results.

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