Breast Cancer Patients with Progesterone Receptor PR-A-Rich Tumors Have Poorer Disease-Free Survival Rates

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ABSTRACT

Purpose: **No study has yet analyzed whether changes in relative expression levels of progesterone receptor (PR) isoforms A and B in human breast tumors have significance in predicting clinical outcome. Human PRs are ligand-activated nuclear transcription factors that mediate progesterone action. Their presence in breast tumors is used to predict functional estrogen receptors (ERs) and, therefore, also to predict the likelihood of response to endocrine therapies and disease prognosis. The two PR isoforms, PR-A and PR-B, possess different *in vitro* and *in vivo* activities, suggesting that in tumors, the ratio of their expression may control hormone responsiveness. In general, PR-B are strong transcriptional activators, whereas PR-A can act as dominant repressors of PR-B and ER. Thus their balance may affect tamoxifen response in breast cancers.**

Experimental Design: **To determine whether differential expression of the PR isoforms is associated with clinical outcome and hormonal responsiveness, PR-A and PR-B were measured by immunoblot analysis of cell lysates from 297 axillary node-positive breast tumors.**

Results: **Expression of the two isoforms correlated with each other, as well as with ER. Additional analyses revealed that patients with PR-positive tumors but high PR-A:PR-B ratios, which were often caused by high PR-A levels, were 2.76 times more likely to relapse than patients with lower ratios, indicating resistance to tamoxifen.**

Conclusions: **This study suggests that knowledge of the PR-A:PR-B ratio may identify a subgroup of ER-positive/PR-positive patients with node-positive breast cancer that benefit poorly from endocrine therapy.**

Received 9/2/03; revised 11/18/03; accepted 11/24/03.
Grant support: NIH/National Cancer Institute R01CA72038 (S. Fuqua), NIH/National Cancer Institute P50 CA58183 (C. Kent Osborne), and DK48238, CA26869, and the Avon Foundation (K. Horwitz).
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INTRODUCTION

In normal mammary glands, progesterone promotes epithelial cell proliferation and is essential for lobulo-alveolar outgrowth (1). This hormone mediates its effects through progesterone receptors (PRs), which belong to a large superfamily of ligand-activated nuclear receptors. Human PR proteins exist as two isoforms, termed PR-A and PR-B, that are transcribed from a single gene under the control of separate promoters (2, 3). Both receptors bind progestins and interact with progesterone response elements (PREs), but there is increasing evidence that they have different functions *in vivo* (4). *In vitro*, PR-B are transcriptional activators of some promoters in a variety of cell types in which PR-A have low activity. PR-A, on the other hand, are dominant repressors of PR-B, estrogen receptors (ERs), and other steroid receptors in a promoter- and cell-type-specific manner (5–8).

Both PRs contain the same functional domains involved in two transcriptional activation functions, DNA binding, dimerization, and ligand binding. The binding of progesterone to both PRs induces conformational changes that lead to the formation of homo- or heterodimers, increased receptor phosphorylation, interaction with target gene promoters by binding to progesterone response elements, and to specific coactivators and general transcription factors (9). The two PR isoforms differ, however, in that PR-A lacks the first 164 NH_2-terminal amino acids contained in PR-B. This NH_2-terminal region encodes a third transactivation function that is specific to PR-B (10), and plays an essential role in the transcriptional regulation of PR-B-specific genes (11).

It is believed that regulated expression of PR-A and PR-B is critical for appropriate mammary gland responsiveness to progesterone. Indeed, in transgenic mice carrying an excess of PR-A, mammary gland development is characterized by disproportionate lateral ductal branching, whereas transgenic mice overexpressing PR-B show inappropriate lobulo-alveolar growth (12, 13). Furthermore, mice in which one or the other PR isoform has been deleted, exhibit developmental mammary gland abnormalities (14). Taken together, these observations show that balanced expression of the two PR isoforms is critical to the control of progesterone responsiveness in normal tissues.

In breast cancers, total PR (as measured by ligand-binding assay) has many of the same prognostic and predictive implications as ER (15, 16). Approximately one-half of primary breast tumors are positive for both PR and ER, whereas less than 5% are negative for ER but still positive for PR. In addition, well-differentiated tumors are more likely to be PR-positive than are poorly differentiated tumors. Several clinical studies have confirmed that elevated total PR levels correlate with an increased probability of response to tamoxifen, longer time to treatment failure, and longer overall survival (17–19).

The classical methods to determine PR levels in breast tumors include various ligand-binding assays on total tumor

Table 1 Distribution of patient characteristics[a]

	Overall (n = 297)	Endocrine-treated (n = 178)	No treatment (n = 119)
Age (yr)			
<50	43 (14%)	19 (11%)	24 (20%)
≥ 50	254 (86%)	159 (89%)	95 (80%)
Tumor size (cm)			
0–2	72 (24%)	49 (27%)	23 (19%)
> 2–5	170 (57%)	101 (57%)	69 (59%)
>5	54 (18%)	28 (16%)	26 (22%)
Missing	1 (−)		1 (−)
Nodes			
1–3	162 (55%)	98 (55%)	64 (54%)
>3	134 (45%)	80 (45%)	54 (46%)
S Phase			
Low (0–<6%)	65 (22%)	45 (26%)	20 (17%)
Intermediate (≥6–≤10%)	97 (33%)	56 (32%)	41 (35%)
High (>10%)	130 (45%)	73 (42%)	57 (48%)
Missing	5 (−)	4 (−)	1 (−)
Ploidy			
Diploid	102 (35%)	59 (34%)	43 (36%)
Aneuploid	191 (65%)	115 (66%)	76 (64%)
Missing	4 (−)	4 (−)	
ER[b] (fmol/mg)			
Positive (≥3)	264 (89%)	168 (94%)	96 (81%)
Negative (0–<3)	33 (11%)	10 (6%)	23 (19%)
PR (fmol/mg)			
Positive (≥5)	174 (61%)	116 (66%)	58 (53%)
Negative (0–<5)	112 (39%)	60 (34%)	52 (47%)
Missing	11 (−)	2 (−)	9 (−)
AIB1, IU, median (range)	1.12 (0.26–5.73)	1.11 (0.26–5.73)	1.17 (0.30–5.40)
HER-2, IU, median (range)	1.00 (0–4)	1.00 (0–4)	1.00 (0–4)
Median follow-up time (mo), median (range)	65 (0–300)	72 (8–257)	50 (0–300)

[a] ER, and P.R. values were previously determined by ligand-binding assays. AIB1 and HER-2 were previously determined by immunoblot analyses.
[b] ER, estrogen receptor; PR, progesterone receptor; IU, intensity unit(s).

extracts, as well as receptor antibody-based assays such as immunohistochemistry (20–23). However, these assays cannot differentiate between the two PR isoforms, so that information about the expression and potential role of each isoform as a prognostic and/or predictive factor is currently not available. Only a few studies, involving small groups of tumors, have separately examined PR-A and PR-B expression (24–26). Collectively, they indicate that expression of both PR isoforms correlates with ER expression, but that the ratios of PR-A ratio to PR-B ratio vary widely, with some tumors expressing a large excess of one or the other isoform. Importantly, these studies did not analyze the role of PR-A and PR-B as predictive factors for endocrine therapies. Therefore, the purpose of the present study was to determine whether differential expression of PR-A and/or PR-B is associated with hormonal responsiveness and clinical outcome in breast cancers. We present for the first time data showing that high PR-A levels identify a subgroup of women with a poorer response to tamoxifen.

MATERIALS AND METHODS

Patient Tumor Specimens

Frozen primary breast tumor specimens from a cohort of 297 axillary lymph node-positive patients were selected from the tumor bank at The Breast Center of Baylor College of Medicine, for use in the immunoblot study. Patient and tumor characteristics are shown in Table 1. Proteins in 30 mg of frozen, pulverized powders were extracted in 300 μl of 5% SDS in distilled water at 90°C for 5 min. Protein concentration was determined using the Pierce bicinchonic acid method; typical yields were 2–5μg/μl. Samples were stored at −70°C.

Cell Line Standard

T47D human breast cancer cells were maintained in MEM with 10% fetal bovine serum and 10^{-8} M insulin. The cells were grown in 100-mm tissue culture dishes and were passaged when they reached 80% confluence. Harvest and extraction involved a single-step process in the culture dish: the cells were washed in the dish twice with cold PBS, and proteins were then extracted with 5% SDS in distilled water at 90°C for 5 min. The extraction mixture was centrifuged (5 min at 13,000 rpm) to pellet the debris, and protein remaining in the supernatant was determined using the Pierce bicinchonic acid method. This standard preparation was stored at −70°C.

Immunoblots

For electrophoresis, 20 μg of extracted protein and sample buffer [0.05 M Tris (pH 6.8), containing 2% SDS, 2.5% β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue as tracking dye) in a total volume of 18 μl were placed in boiling water for 2 min, cooled to room temperature, and centrifuged 1

min. The T47D reference standards (5 μg and 10 μg of protein in 18 μl/gel) were included on each gel as positive controls. Proteins were resolved by electrophoresis on precast 8% Tricine-Glycine polyacrylamide gels (Invitrogen, Carlsbad, CA) and were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The blots were rinsed 5 min with PBS containing 0.1% Tween 20 (PBST). After blocking with 5% nonfat dry milk in PBST, the blots were incubated for 1 h in the primary antibody (1:2000 mouse anti-PgR1294; Dako, Carpinteria, CA) in 5% nonfat dry milk in PBST. PgR-1294 specifically recognizes both PR-A and PR-B isoforms on immunoblots, and was selected from among several commercially available antibodies because of its superior sensitivity. The blots were washed three times in PBST, followed by incubation for 1 h in 5% nonfat dry milk in PBST and 1:5000 horseradish peroxidase-labeled anti-mouse immunoglobulin (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were washed five times in PBST, after which the labeled protein was visualized on a FluorChem digital imaging system (Alpha Innotech, San Leandro, CA) using an enhanced chemiluminescence detection system. Band intensities were measured densitometrically using the AlphaEaseFC software (Alpha Innotech). PR-A and PR-B levels in tumors were normalized to PR-B levels in the T47D positive control lysate (10 μg) from the same immunoblot. The purpose of normalization was to provide a measure of each isoform relative to a similar reference protein standard across the entire study, and to use the resulting number to order the tumor samples from lowest to highest PR-A and PR-B levels.

Other Factors

Several molecular markers have been previously measured on the same samples used in this study. Total ER and PR were measured by ligand-binding assay as described elsewhere (27), whereas S-fraction was calculated by flow cytometry (28). AIB1 and HER-2 levels were determined by immunoblot analyses as described previously and are expressed as normalized intensity units 29, 30). AIB-1 was used as a continuous variable in subsequent statistical analyses. HER-2 was measured on an ordinal scale (0−4) with higher values correlated with shorter disease-free survival (30).

Statistical Methods

Descriptive Analysis. Clinical characteristics, PR isoforms, and selected markers were summarized separately in tamoxifen-treated and untreated patients using descriptive statistics. Correlations between PR isoforms and patient clinical characteristics or other molecular markers were evaluated using Spearman's rank correlation (*r*). Spearman's correlation ranges in value from −1 to +1. A value of 0 indicates no association, whereas values near +1 or −1 indicate strong positive or negative relationships, respectively. All of the variables in the correlation analysis were analyzed as continuous variables.

Univariate Analysis of Disease-Free Survival and Overall Survival. The disease-free interval was calculated from the date of diagnosis to date of first recurrence or first metastasis (local or distant). Patients without recurrence were censored at the time of last follow-up or death. Overall survival was calculated from the date of diagnosis to date of death from any cause. Patients who were alive at the last follow-up were censored at the last follow-up date. Overall survival included all deaths regardless of cause because cause of death data, especially if reported as "not due to breast cancer," are sometimes unreliable. This definition of overall survival is the most conservative estimate of patient outcome. Therefore, disease-free survival was the primary outcome sought in this analysis and has more relevance than overall survival. PR-A, PR-B, and PR-A:PR-B ratio were dichotomized (0 to <1 *versus* ≥ 1 for PR-A and PR-B; 0 to ≤ 1 *versus* >1 for PR-A:PR-B ratios) for inclusion in survival analyses. Two criteria were used to determine this cut point before performing univariate or multivariate analyses of the association of dichotomized levels of PR-A, PR-B, or PR-A:PR-B with patient outcome. The first statistical method was based on evaluating the functional forms of the quantitative levels of PR-A, PR-B, and PR-A:PR-B using Martingale residuals (31). In brief, this method fits a Cox proportional hazards model with only an intercept term in the model (*i.e.,* no covariates). The Martingale residuals from this intercept-only model were plotted against quantitative levels of each of PR-A, PR-B, and PR-A:PR-B. A reasonably linear plot indicates that a variable can be entered as a continuous variable in a model, whereas a nonlinear plot indicates some threshold effect. The cut points for PR-A, PR-B, and PR-A:PR-B were chosen based on where the threshold effect is evident from the plots. Secondly, the proposed dichotomization cut point was approximately equal to the median value of the distribution of the quantitative levels of PR-A, PR-B, PR-A:PR-B values. Thus, although the cut points are arbitrary, they are selected based on statistical methods performed *a priori* to our analyses. Disease-free survival and overall survival were estimated using the Kaplan-Meier method and compared using the Wilcoxon test, which is more appropriate than the log-rank test when the assumption of proportional hazards is violated (see *Multivariate Cox Regression Model for Disease-Free Survival and Overall Survival*). Analysis was performed separately for treated and untreated patients.

Multivariate Cox Regression Model for Disease-Free Survival and Overall Survival. PR-A and PR-B are highly correlated and were, therefore, analyzed separately to examine their individual effects. In addition, all multivariable Cox regression modeling was carried out separately for treated and untreated patients. Clinical characteristics (patient age, tumor size, nodes, S phase, and ploidy) were dichotomized or trichotomized according to standard cut-offs, as indicated in Table 1, and were coded ordinally (*i.e.,* 0, 1, 2) for inclusion as continuous variables in the model. The molecular markers, AIB1 and HER-2, were entered as continuous variables in the model.

Previous studies have reported the nonproportional effect of ER on disease-free survival and overall survival (32). For this specific patient population, tests of the proportionality assumption revealed significant departures from proportionality for both PR-A and PR-B but not for ER. Testing was accomplished by including each marker in the model as both fixed and time-dependent variables. Plots of time-varying regression coefficients allowed us to visualize the nature of the nonproportionality and to determine an appropriate method of adjustment. To account for nonproportionality, Cox models included two terms for each of the PR isoforms, one to account for effects before 5 years and another to account for effects beyond 5 years

of follow-up. Cox regression models were constructed using forward stepwise selection with the level of significance to enter or stay in the model set at 0.05.

RESULTS

PR-A and PR-B Expression and Their Relationship to Other Clinical Markers. Because recent publications suggest that PR-specific antibodies might differ in their ability to recognize PR-A and PR-B levels (22–26), we tested various commercial antibodies currently used to assess PR in clinical breast cancer specimens. As shown on a representative immunoblot in Fig. 1, both of the clinically used antibodies PgR-1294 and PgR-636 recognized similar PR-A:PR-B ratios in cell extracts from MCF-7, T47D, and ZR75 breast cancer cells. However, PgR-1294 was by far the most sensitive antibody tested in Western blot analysis. We, therefore, used only PgR-1294 to detect PR-A and PR-B in tumor extracts.

PR-A and PR-B levels in SDS protein extracts from 297 tumor specimens from patients with operable stage II breast cancers with positive axillary nodes were analyzed by immunoblotting with the PgR-1294 antibody. This approach was chosen because there are currently no antibodies available that distinguish between PR-A and PR-B by immunohistochemistry. Additionally, because it has never been reported that breast tumor stromal infiltrates express PR, the use of immunoblotting to measure PR isoforms is not only justified but is also the only method available for explorative analyses of the two PR isoforms. The study population consisted of two subsets: (*a*) 119 patients who received no adjuvant therapy after primary treatment; and (*b*) 178 patients treated with only tamoxifen. The majority of patients were above age 50. Most of the tumors were smaller than 5 cm, with intermediate to high S phase. By study design, all of the patients had positive lymph nodes. Approximately 89% of the tumors expressed ER, whereas 61% were positive for PR, as determined by ligand-binding assay at the time of diagnosis. Even though PR levels in tumor tissues of less than 5 fmol/mg cytosolic protein are considered negative, many of these breast cancers still contain some amount of PR. Therefore, tumors that were classified as "PR-negative" by ligand-binding assay, were also included in this study. However, those defined as PR-positive by ligand-binding assay were also analyzed separately (see Fig. 4). Median scores for AIB1 and Her-2 were 1.12 intensity unit and 1.00 intensity unit, respectively, as determined by immunoblot analysis. Overall median follow-up was 65 months.

PR-A and PR-B levels were semiquantitatively calculated by measuring the band intensities on immunoblots normalized to band intensity of PR-B from T47D cell extracts run on the same gel (a representative gel is shown in Fig. 2). This normalization procedure provided a baseline protein standard (T47D PR-B levels) across the entire set of electrophoretic gels run for the study. Expression of the two PR isoforms was heterogeneous among the 297 tumors and ranged for PR-A from a band intensity of 0 to a maximum of 52 intensity units and for PR-B from 0 to 45 intensity units, respectively (Table 2). There were 12 tumors that expressed only PR-B in which PR-A was equal to 0, 7 tumors that expressed only PR-A in which PR-B was equal to 0, and 11 tumors in which both PR-A and PR-B were equal to 0. Thus, of the 112 tumors that were classified as PR-negative by ligand-binding assay, only 11 were completely negative by immunoblotting. The PR-A:PR-B protein ratio ranged from 0 to a maximum of 31 with 72% of the tumors having a PR-A:PR-B ratio between 0.5 and 2. A ratio of 0.5 indicates a 2-fold excess of PR-B over PR-A, whereas a ratio of 2 indicates a 2-fold excess of PR-A over PR-B. Only a small number of the patients had breast tumors with more than a 2-fold excess of PR-B over PR-A (18%), or more than 2-fold excess of PR-A over PR-B (10%). The median band intensity values were 0.72 and 0.84 for PR-A and PR-B, respectively, and median PR-A:PR-B ratio was 0.96.

We then correlated PR-A and PR-B levels with various biological markers and clinical variables. In these tumors, PR-A and PR-B protein expression were highly correlated with each other ($r = 0.91$; $P < 0.0001$) and with total PR as measured by ligand-binding assay ($r = 0.60$; $P < 0.0001$; Table 3). The high correlation of the PR isoforms with each other suggests that, in this patient population, most breast tumors expressed similar levels of PR-A and PR-B. Both isoforms were also significantly

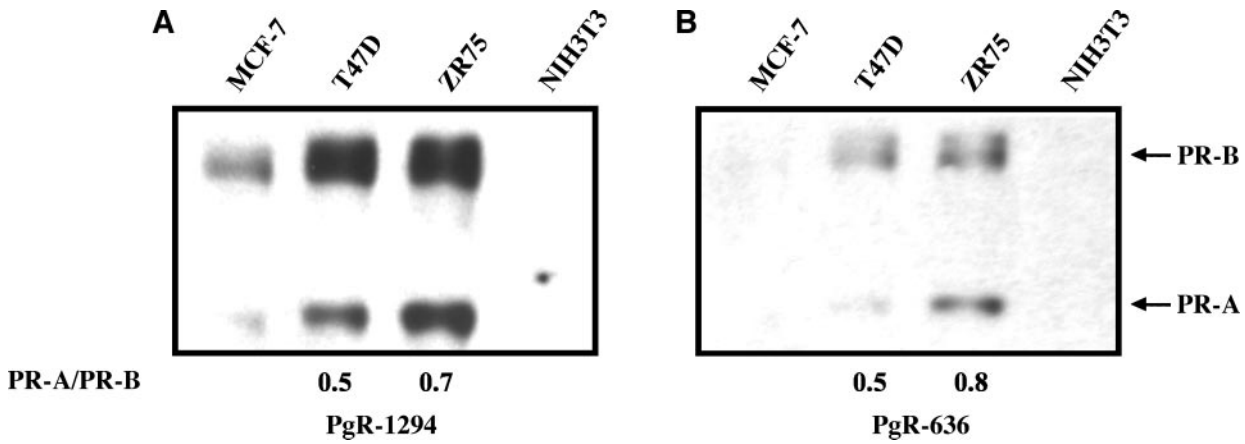

Fig. 1 Immunoblot analysis of progesterone receptors, PR-A and PR-B, with antibodies PgR-1294 (*A*) and PgR-636 (*B*). Equal amounts of total protein from MCF-7, T47D, and ZR75 breast cancer cells as well as total protein from NIH3T3 cells (negative control) were analyzed.

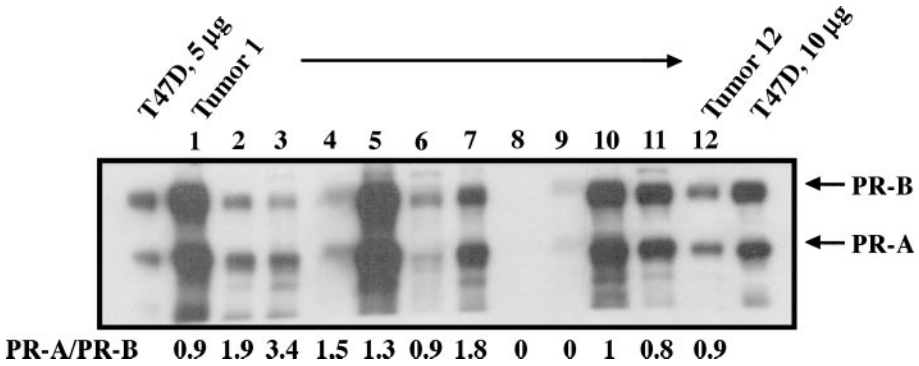

Fig. 2 Representative example of progesterone receptors PR-A and PR-B immunoblots from 12 tumor lysates. First and last lanes are the standards used for normalization containing 5 and 10 μg of T47D cell extracts, respectively.

correlated with ER ($P < 0.0001$), agreeing with the general observation that estrogens induce both PR-A and PR-B (2). The PR isoforms also showed a significant inverse correlation with tumor size, S phase, and number of positive nodes. It has been suggested that the ratio of expression of PR-A and PR-B may be even more important than the absolute levels of PR. In these breast cancers, PR-A:PR-B ratios and PR-A were significantly correlated ($P < 0.0001$), indicating that high ratios are an effect of excess PR-A rather than low PR-B levels (Table 3).

Disease-Free Survival and Overall Survival by PR Isoforms. Comparisons of disease-free survival among 178 tamoxifen-treated patients with 0 to <1 *versus* ≥1 levels of PR-A and PR-B are shown in Fig. 3, *A* and *B*. PR-A, PR-B, and PR-A:PR-B ratios were dichotomized into appropriate equal-sized groups (0 to <1 *versus* ≥1 for PR-A and PR-B; 0 to ≤1 *versus* >1 for PR-A:PR-B ratios) because plots of Martingale residuals confirm that the relationship between disease-free survival or overall survival is reasonably well represented by this dichotomization. Patients with tumors that expressed PR-A but not PR-B were assigned into the PR-A-rich category.

When each isoform was analyzed separately, treated patients with lower intensity values (0 to <1) of PR-A or PR-B had a significantly poorer disease-free survival than those with higher values (≥ 1; $P = 0.0056$ and 0.0011, for PR-A and PR-B, respectively). Both isoforms were also significantly predictive of overall survival (Fig. 3, *D* and *E*) among treated patients. We also analyzed the effect of the PR-A:PR-B protein ratios on disease-free survival and overall survival in these patients, regardless of whether they were classified as PR-negative or PR-positive by ligand-binding assay. Comparisons were done among treated patients with 0 to ≤1 (PR-A-poor) *versus* >1 (PR-A-rich) PR-A:PR-B ratios (Fig. 3, *C* and *F*). We found that patients who were not selected for PR status as determined by ligand-binding assay and whose tumors were PR-A rich had a somewhat poorer, but not statistically significant disease-free survival than patients with lower ratios ($P = 0.12$). There was no association between PR-A:PR-B ratios and overall survival. We also compared disease-free survival among treated patients whose tumors had an excess of PR-B (>0 to ≤0.5), similar levels of PR-A and PR-B (>0.5 to <2), or an excess of PR-A (≥2), but found no association (data not shown). We did not detect significant effects of PR-A, PR-B, or PR-A:PR-B ratio on disease-free survival or overall survival among untreated patients (data not shown).

Table 2 Distribution progesterone receptors (PRs), PR-A, PR-B, and PR-A:PR-B ratio in study population[a]

	Overall ($n = 297$)	Endocrine-treated ($n = 178$)	No treatment ($n = 119$)
PR-A,[b] IU[c]	0.72 (0–52)	0.76 (0–26)	0.72 (0–52)
PR-B,[b] IU	0.84 (0–45)	1.10 (0–23)	0.64 (0–45)
PR-A:PR-B	0.96 (0–31)	0.86 (0–31)	1.07 (0–30)

[a] Values shown are medians and, in parentheses, range.
[b] PR-A and PR-B values normalized to PR-B band of T47D control.
[c] IU, intensity unit(s).

Multivariate Cox Regression Analysis of Disease-Free Survival and Overall Survival. The predictive effect of PR-A and PR-B on disease-free survival in tamoxifen-treated patients is shown in Table 4. A model that includes PR-A along with the number of positive nodes and AIB1 shows that this isoform is strongly associated with disease-free survival in treated patients (Table 4). Specifically, patients with lower PR-A values are 2.39 times more likely to relapse than patients with higher PR-A values [95% confidence interval (CI), 1.33–4.29]. However, this effect disappears beyond 5 years of follow-up (hazard ratio, 0.53; 95% CI, 0.16–1.73). PR-B have a similar effect on patient relapse during the first 5 years of follow-up (hazard ratio, 2.49; 95% CI, 1.41–4.40; Table 4). Number of positive nodes and high AIB1 levels were also significantly associated with patient relapse along with PR-B. Neither isoform was significantly associated with disease-free survival among the untreated patients (data not shown).

Similar to disease-free survival, both PR isoforms were significantly associated with overall survival (Tables 5 and 6). Specifically, patients with lower PR-A values are 2.81 times more likely to die than patients with higher PR-A values (95% CI, 1.58–5.02). However, this effect appears to weaken beyond 5 years of follow-up (hazard ratio, 1.41; 95% CI, 0.86–2.30). PR-B has a similar, although slightly lesser effect than PR-A on overall survival (hazard ratio, 2.41; 95% CI, 1.39–4.17; Table 6). Similar to PR-A, this effect becomes less evident beyond 5 years of follow-up. Larger tumor size and high AIB1 levels were also significantly predictive of overall survival. Neither PR-A nor PR-B were associated with overall survival among the untreated patients (data not shown).

Table 3 Spearman rank correlation of progesterone receptors (PRs), PR-A and PR-B, with other variables

Variable	PR-A correlation (*P*)	PR-B correlation (*P*)	PR-A:PR-B correlation (*P*)
PR-A		0.91 (<0.0001)	0.31 (<0.0001)
PR-B	0.91 (<0.0001)		−0.03 (0.617)
PR[a]	0.60 (<0.0001)	0.60 (<0.0001)	0.12 (0.032)
ER[a]	0.32 (<0.0001)	0.36 (<0.0001)	−0.05 (0.401)
Age	0.03 (0.596)	0.09 (0.133)	−0.12 (0.048)
Tumor size	−0.12 (0.033)	−0.14 (0.016)	0.043 (0.458)
Nodes	−0.14 (0.016)	−0.15 (0.011)	−0.034 (0.558)
S phase	−0.16 (0.005)	−0.15 (0.010)	−0.030 (0.608)
AIB1	−0.02 (0.751)	−0.04 (0.515)	−0.028 (0.728)
HER-2	0.06 (0.322)	0.06 (0.260)	−0.063 (0.278)

[a] ER and PR were previously determined by ligand-binding assays.

Association of PR-A:PR-B Ratios with Disease-Free Survival in the Subset of PR-Positive Patients as Defined by Ligand-Binding Assays. Knowledge of the total PR status as determined by ligand-binding assay, in addition to the ER status, has been reported to improve the prediction of response to tamoxifen therapy in breast cancer patients (17). To determine whether information about PR-A:PR-B ratios in the PR-positive subset as defined by ligand-binding assay might improve prediction of response to tamoxifen, we compared disease-free survival among 116 tamoxifen-treated, PR-positive patients with PR-A:PR-B ratios of 0 to ≤1 (75 patients) *versus* ones with PR-A:PR-B ratios >1 (41 patients; Fig. 4). Treated patients with higher PR-A:PR-B ratios (>1) had a significantly poorer disease-free survival than those with lower ratios (0 to ≤1; $P = 0.0209$). Of note, we observed that ~45% of all combined ER-positive/PR-positive patients in this study (treated and untreated) express the potential high-risk phenotype [PR-A:PR-B ratio >10 (data not shown)]. A multivariate Cox regression model including PR-A:PR-B ratio along with tumor size and AIB1 shows that patients with higher ratios (>1) are 2.76 times more likely to relapse than patients with lower ratios (95% CI, 1.43–5.33; $P = 0.0024$). On the other hand, HER-2 expression, an important molecular marker previously shown to predict tamoxifen resistance (30), was not statistically significant for outcome in this PR-positive subgroup. This suggests that knowledge of the PR-A:PR-B ratios in PR-positive breast cancers improves prediction of patient response to tamoxifen treatment and identifies a subgroup of patients who may benefit from other treatment strategies. It must be stressed that other currently available markers fail to identify these patients.

DISCUSSION

PR have been extensively studied in breast cancer; positive total PR status correlates with favorable prognostic features and is used clinically to identify patients who are likely to benefit from hormonal therapies (33). PR exists as two isoforms, called PR-A and PR-B, which have distinct roles in regulating the effects of progesterone (4). PR levels in breast tumors were traditionally measured by various types of ligand-binding assays, and are currently assessed by immunohistochemistry, but neither approach is able to discriminate between PR-A and PR-B. In the present study, PR-A and PR-B were measured by immunoblotting of tumor lysates from patients with node-positive primary breast cancers; and their independent levels, as well as PR-A:PR-B ratios, were correlated with molecular tumor markers and clinical outcome. Our results provide the first direct demonstration that the loss of coordinate expression of PR-A and PR-B impacts the clinical treatment response of breast cancer patients.

Specifically, we found that, in PR-positive patients who had node-positive primary breast cancers and who received local therapy followed by adjuvant tamoxifen, high PR-A:PR-B ratios predicted shorter disease-free survival, indicating resistance to tamoxifen in both univariate and multivariate analyses. This indicated either a higher level of intrinsic (*de novo*) resistance, or a more rapid onset of acquired resistance in PR-A rich tumors. There is increasing evidence that changes in the PR-A:PR-B ratio, in particular PR-A overexpression, can alter cellular properties of breast cancer cells, such as cell–cell adhesion and responses to progestins (34). Additionally, on promoters in which ligand bound PR-A have low or no activity, these receptors can act as a dominant repressors of PR-B and ERs (5, 6, 8, 35). It has also been reported that, for unknown reasons, about 25% of ER-positive/PR-positive breast tumors fail to respond to tamoxifen therapy (36). We now postulate that overexpression of PR-A could affect the response of ER to tamoxifen, the most commonly used hormonal agent. Indeed, data from the subgroup of tumors that are PR-positive by ligand-binding assay indicate that high PR-A:PR-B ratios are associated with failure of tamoxifen treatment as indicated by shorter disease-free survival (Fig. 4). Furthermore, our study also indicates that the high PR-A:PR-B ratios, at least in this study population, were frequently caused by excess PR-A, rather than low PR-B. Predominance of PR-A could cause tamoxifen resistance by directly repressing the transcriptional activity of ER as suggested by several *in vitro* studies (5, 6, 8, 35), or indirectly by PR-A-directed up-regulation of genes known to be involved in tumor aggressiveness or prognosis. Alternatively, one could argue that any dominant-negative inhibitor of ER might exert additive growth-inhibitor effects with tamoxifen use, because both approaches share a common target, ER. However, this is not what we observed in this patient cohort. It is conceivable that excess PR-A could have deleterious effects on tamoxifen response and hormonal signaling, independent of its effects on ER, or indirectly through effects on its own downstream signaling molecules that may converge on the ER hormone response network. Despite the strong effect of PR-A:PR-B ratio in the subset of endocrine-treated, PR-positive patients that we studied, a larger

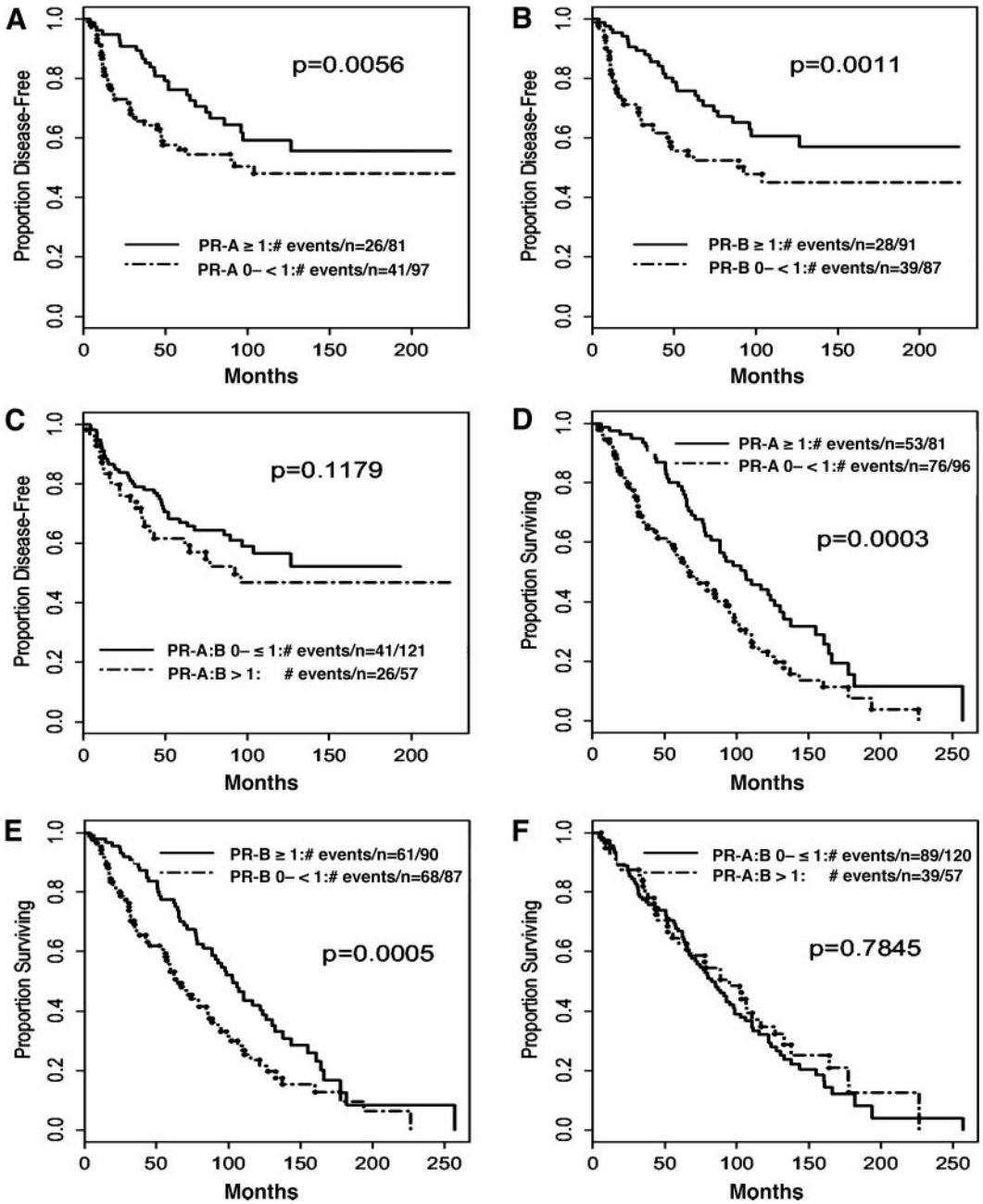

Fig. 3 Disease-free and overall survival (Kaplan-Meier) for tamoxifen-treated patients stratified by levels of progesterone receptors PR-A (*A* and *D*), PR-B (*B* and *E*), and PR-A:PR-B protein ratios (*C* and *F*). The number of events in each group, and *P* values (*p*) are also shown.

study is warranted to confirm our findings on this patient subgroup, and biological experiments are needed to explore the potential association between PR-A and response to antiestrogens.

The frequency of excess PR-A in our study is in agreement with another study showing a predominance of the PR-A isoform in 39% (15 of 39) of invasive breast cancers, and 40% (6 of 15) of ductal carcinomas *in situ* using dual-immunofluorescence (37). However, in contrast to our study, in which we found no association with tumor size, are results that have been obtained using T47D tumor xenografts (38). In this latter study, the authors demonstrate that PR-A tumors without PR-B expression exhibit slower growth, compared with tumors expressing only PR-B. Furthermore, PR-A-only-expressing tumors responded to an 8-week treatment with tamoxifen. However, it is very difficult to generalize and extrapolate data obtained from an animal model to human tumors. It is possible that the differences could be explained by the single fact that the Sartorius *et al.* study (38), used cells that express exclusively only one or the other PR isoform. In our human tumor study, virtually every

tumor expressed at least some level of both receptor isoforms. Furthermore, the treatment duration time in the animal model is much shorter than any the patients received, and is certainly not designed to represent a tamoxifen "resistance" protocol.

We did not observe a significant association between PR isoform levels, and either AIB or HER-2 levels, nor was HER-2 a significant predictive factor in our multivariate analyses in treated patients, although there is ample evidence that HER-2 is a predictor of tamoxifen resistance (39, 40). In our multivariate model, there was a weak association of high HER-2 levels with tamoxifen resistance, but HER-2 subsequently dropped out of the final model in this specific patient cohort. In a recent study (29), Kaplan-Meier estimates of disease-free survival of patients expressing both AIB and HER-2 were predictive of response to endocrine therapy. The discrepancy between our results and those of others could be influenced by the fact that our study population was entirely node-positive and also by the fact that we used immunoblot analysis as compared with immunohistochemical or fluorescence *in situ* hybridization methods, which are most prevalently used for clinical HER-2 measurements. Also of note is our finding that levels of HER-2 are significantly higher in ER-positive/PR-A-positive xenografts, than in ER-positive/PR-B-positive xenografts,[6] which is consistent with Bamburger *et al.* (25), who showed that PR-B levels are correlated with absent-to-low expression of HER-2. Thus, it remains an intriguing question as to whether PR can exert a direct effect on HER-2.

Table 4 Cox regression model of progesterone receptors (PRs), PR-A and PR-B, with disease-free survival: tamoxifen-treated group

Variable	HR[a] (95% CI)	*P*
PR-A		0.0052
< 5 years		
≥1 IU	1.00	
0–<1 IU	2.39 (1.33–4.29)	
> 5 years		
≥1 IU	1.00	
0–<1 IU	0.53 (0.16–1.73)	
Node group		
1–3	1.00	
> 3	2.57 (1.56–4.24)	0.0002
AIB1	1.50 (1.10–2.04)	0.0103
PR-B		0.0039
< 5 years		
≥1 IU	1.00	
0–<1 IU	2.49 (1.41–4.40)	
> 5 years		
≥1 IU	1.00	
0–<1 IU	0.65 (0.20–2.13)	
Node group		
1–3	1.00	
> 3	2.51 (1.52–4.14)	0.0003
AIB1	1.51 (1.11–2.05)	0.0083

[a] HR, hazard ratio; CI, confidence interval; IU, intensity unit(s).

Table 5 Cox regression model of progesterone receptor (PR) PR-A with overall survival: tamoxifen-treated group

Variable	HR[a] (95% CI)	*P*
< 5 years		0.0004
≥1 IU	1.00	
0–<1 IU	2.81 (1.58–5.02)	
≥ 5 years		
≥1 IU	1.00	
0–<1 IU	1.41 (0.86–2.30)	
Tumor size group		0.005
0–2 cm	1.00	
≥2–5 cm	1.51 (1.13–2.00)	
>5 cm	2.26 (1.28–4.01)	
AIB1	1.26 (1.01–1.58)	0.041

[a] HR, hazard ratio; CI, confidence interval; IU, intensity unit(s).

Table 6 Cox regression model of progesterone receptor (PR) PR-B with overall survival: endocrine-treated group

Variable	HR[a] (95% CI)	*P*
< 5 years		0.005
≥1 IU	1.00	
0–<1 IU	2.41 (1.39–4.17)	
≥ 5 years		
≥1 IU	1.00	
0–<1 IU	1.12 (0.69–1.81)	
Tumor size group		0.005
0–2 cm	1.00	
>2–5 cm	1.50 (1.13–1.98)	
>5 cm	2.24 (1.28–3.94)	

[a] HR, hazard ratio; CI, confidence interval; IU, intensity unit(s).

A current model of breast tumorigenesis suggests that breast cancers evolve from normal human breast epithelium through a series of histologically defined abnormalities starting with typical hyperplasia, to primary invasive disease, and finally to metastatic breast cancer. Not all stages of the disease are believed to be obligatory, allowing a number of possible pathways for disease progression. The importance of PR for the initiation of mammary tumor formation was recently highlighted by a study showing a significant reduction in carcinogen-induced tumorigenesis in PR knockout mice (41). Unlike the rodent mammary gland, the normal human breast coexpresses PR-A and PR-B at similar levels within the same cells throughout the menstrual cycle (42). It is, therefore, thought that coordinate expression of PR-A and PR-B is required for the normal progesterone responses of the mammary gland, and that deregulation of this ratio appears early in tumorigenesis (24, 37). Our observation of tamoxifen resistance in tumors with high PR-A levels indicates that the loss of coordinate PR isoform expression results not only in perturbation of PR signaling but could also indicate in alteration of ER signaling.

In this cohort of breast cancer patients, expression of both PR isoforms was also correlated with total PR and ER as measured by ligand-binding assays. Correlation with ER has also been observed in another study (25), and agrees with the observation that estrogen induces both PR-A and PR-B isoform expression (2). As in another study analyzing PR-A and PR-B expression in breast cancers (24), protein levels of the two PR isoforms in our study population were highly correlated with each other. Furthermore, we also found that in tamoxifen-treated patients, high levels of either PR-A or PR-B predicted longer disease-free survival and overall survival in both univariate and

[6] Unpublished observations.

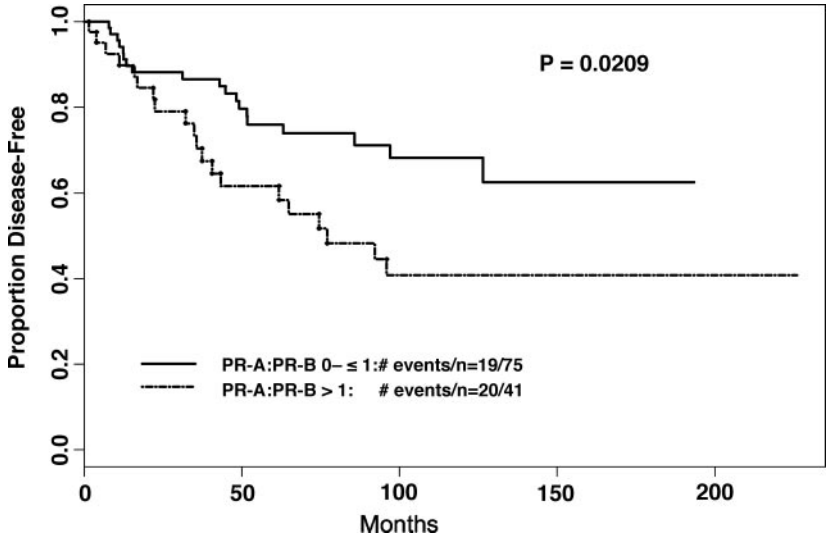

Fig. 4 Disease-free (Kaplan-Meier) for tamoxifen-treated PR-positive patients stratified by PR-A/PR-B protein. The number of events in each group, and *P* values (*p*) are also shown.

multivariate analyses; neither PR-A nor PR-B predicted the outcome in untreated patients. These results are consistent with numerous studies assessing total PR by ligand-binding assays and immunohistochemistry, which show that total PR levels correlate with favorable tamoxifen response, longer time to treatment failure, and longer overall survival (16).

The magnitude of the hazard ratios showing the effect of PR-A or PR-B within 5 years of follow-up range from 2.4 to 2.8, and is similar to the effect of ER on patient outcome (32). In addition, a number of studies have reported the nonproportional effect of ER on disease-free survival and overall survival (32, 43–45). Thus, the prognostic value of ER appears to fade after long-term follow-up, analogous to the effect we report here for PR. The time-varying effects of other biological factors have also been reported (46). The models presented in Tables 4, 5, and 6, all included statistically significant negative interaction terms between PR-A or PR-B, and time of follow-up. Consequently, these negative interaction terms contributed to the reduction in magnitude of the hazard ratios of both isoforms when calculated beyond 5 years of follow-up. Furthermore, the interaction terms are larger in direction in the disease-free survival model, compared with the overall survival model. Thus, the hazard ratios reverse in direction in the disease-free survival model. One possible interpretation is that PR-positivity correlates with slow growth, so that, early on, PR appears to be associated with fewer distant recurrences. But after 5 years, these patients catch up, and by 10 years, the same number of either PR-positive or PR-negative tumors have recurred. Alternative biological interpretations could also include the emergence of tumors with mutations in PR, or the appearance of clonal variations as tumors progress. The exact mechanisms for the fading of the clinical utility of PR-A, PR-B, and ER, as well as other prognostic factors with long-term follow-up, is currently not known.

In summary, our study suggests that PR-positive patients with high PR-A:PR-B ratios in their breast cancers respond poorly to tamoxifen treatment, and may benefit from other treatment modalities. This particular subgroup of patients with tumors that are PR-A rich would not have been identified in the clinical assessment of breast cancer using present methods that only detect total PR levels. The loss of coordinate expression of PR-A and PR-B, in particular higher levels of PR-A relative to PR-B in tumors, might cause tamoxifen resistance by directly repressing the transcriptional activity of ER or indirectly by PR-A-directed up-regulation of genes known to be involved in tumor aggressiveness or poor prognosis. Undoubtedly a larger study is needed to confirm this exploratory analysis and its clinical implications, because no single predictive study warrants a change in clinical practice. However, because a large percentage of ER-positive/PR-positive might fall into a potential high-risk group (excess PR-A), and the magnitude of the effect is similar to that reported for ER, others should be encouraged to validate these preliminary findings. Similarly, the development of more clinically applicable assays, such as immunohistochemistry, that are capable of specifically assessing the two PR isoforms in clinical samples may be warranted.

ACKNOWLEDGMENTS

We thank G. Chamness for discussion and critical review of the manuscript.

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Clin Cancer Res 2004;10:2751-2760.