**Progesterone Receptor Isoform-Specific Promoter Methylation: Association of PRA Promoter Methylation with Worse Outcome in Breast Cancer Patients**

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**Abstract**

**Purpose:** ERα and PR levels are critical determinants for breast cancer prognosis and response to endocrine therapy. Although PR is known to be silenced by methylation of its promoter, few studies have correlated methylation with PR levels and outcome in breast cancer. There is only one previous small study comparing methylation of the two PR isoforms, PRA and PRB, which are expressed from different promoters, and finally, there is no prior knowledge of associations between isoform-specific methylation and outcome.

**Experimental Design:** We conducted a cohort-based study to test for associations between PRA and PRB methylation, expression, and clinical outcome in tamoxifen-treated patients (n = 500), and in patients who underwent surgery only (n = 500). Methylation and PR levels were measured by bisulfite pyrosequencing and ligand-binding assay, respectively.

**Results:** Low PR levels were significantly associated with worse outcome in all patients. PRA and PRB promoters were methylated in 9.6% and 14.1% of the breast tumors, respectively. The majority (74%) of PR-negative tumors were not methylated despite the significant inverse correlation of methylation and PR levels. PRA methylation was significantly associated with PRB methylation, although a subset of tumors had PRA only (3.9%) or PRB only (8.3%) methylated. Methylation of PRA, but not PRB, was significantly associated with worse outcome in the tamoxifen-treated group.

**Conclusions:** Mechanisms other than promoter methylation may be more dominant for loss of PR. Isoform-specific methylation events suggest independent regulation of PRA and PRB. Finally, this article shows for the first time that PRA methylation plays a unique role in tamoxifen-resistant breast cancer.

**Introduction**

PR is a classical estrogen-regulated gene (1, 2) and is frequently used as a surrogate marker for functional ERα activity. PR exists in 2 isoforms, PRA and PRB, which are the result of transcription from 2 alternative promoters, and initiation of translation at 2 different AUG codons (3).

Structurally, PRB differs from PRA only in that the receptor contains an additional 164 amino acids at the N-terminus of the protein (4). Despite structural similarities, PRA and PRB possess different functional activities. PRB has been found to be a stronger transcriptional activator than PRA, due in part to a third activation domain (AF-3) within the N-terminal 164 amino acids (5). On the other hand, PRA has been shown to act as a repressor which can inhibit other receptors, including ER and PRB (6). PRA and PRB regulate different sets of genes—of 94 progesterone-regulated genes, 65 were uniquely regulated by PRB, 4 uniquely by PRA, and only 25 by both (7). Moreover, the unliganded PR can regulate gene transcription, with PRA being the more active form (8).

This partially independent action of the 2 isoforms is supported by in vivo results: Selective knockout of PRB lead to reduced mammary ductal morphogenesis (9), whereas PRA null mice exhibited normal mammary gland development (10). PRA null mice however display severe phenotypes in the uterus, with defective stromal decidualization, disrupted uterine receptivity, and abnormal progesterone-dependent induction of hyperplasia (11). Transgenic mice with an excess of PRA, or PRB, show abnormal mammary gland development—glands of PRA mice are characterized by disproportionate lateral ductal...
branching, whereas glands from PRB transgenic mice have inappropriate lobuloalveolar growth (12). Taken together, these observations suggest that the balanced expression of the PRA and PRB isoforms is critical for progesterone responsiveness in normal tissues.

Although PRA and PRB are coexpressed in equimolar ratios in the adult normal breast, the ratio is increasingly deregulated as breast cancers progress (13). PRA excess has been associated with poor clinical outcome with more rapid disease recurrence after tamoxifen treatment (14). Wargon and colleagues recently showed that acquired antiprogestin-resistant mammary gland tumors in mice have low levels of PRA and suggested that other mechanisms may be more dominant for loss of PR in the majority of ER-positive/PR-negative tumors. Considering that ER-positive/PR-negative tumors generally have a worse outcome compared with ER-positive/PR-positive tumors, it is critical to decipher those mechanisms in more detail to identify novel treatment targets.

Our analysis of PRA and PRB promoter methylation provides 2 critical results which might ultimately help breast cancer patients, especially with respect to endocrine treatment. First, we discovered a unique association between PRA methylation and tamoxifen response in ER-positive breast tumors, suggesting isoform-specific functions of PR in endocrine treatment response. These data suggest that current and future drug development, aimed at inactivating PR in breast tumors, should consider unique function by PRA and PRB, and thus the generation of isoform-specific drugs, if possible. Second, we show that promoter methylation accounts for loss of PR in only a small subset of PR-negative breast tumors, suggesting that other mechanisms may be more dominant for loss of PR in the majority of ER-positive/PR-negative tumors. Considering that ER-positive/PR-negative tumors generally have a worse outcome compared with ER-positive/PR-positive tumors, it is critical to decipher those mechanisms in more detail to identify novel treatment targets.

Translational Relevance

Our analysis of PRA and PRB promoter methylation provides 2 critical results which might ultimately help breast cancer patients, especially with respect to endocrine treatment. First, we discovered a unique association between PRA methylation and tamoxifen response in ER-positive breast tumors, suggesting isoform-specific functions of PR in endocrine treatment response. These data suggest that current and future drug development, aimed at inactivating PR in breast tumors, should consider unique function by PRA and PRB, and thus the generation of isoform-specific drugs, if possible. Second, we show that promoter methylation accounts for loss of PR in only a small subset of PR-negative breast tumors, suggesting that other mechanisms may be more dominant for loss of PR in the majority of ER-positive/PR-negative tumors. Considering that ER-positive/PR-negative tumors generally have a worse outcome compared with ER-positive/PR-positive tumors, it is critical to decipher those mechanisms in more detail to identify novel treatment targets.

Materials and Methods

Study population

The Breast Center at Baylor College of Medicine (BCM; Houston, TX) maintains 2 databases of breast cancer patients whose biopsy or mastectomy specimens were sent to central laboratories for steroid receptor assays. The first database was funded by the National Cancer Institute (NCI; Bethesda, MD) as part of a Program Project grant and is designated the PPG/P01 database. All receptor assays were conducted in a central laboratory at UT Health Science Center (San Antonio, TX). The second database was funded by the NCI as part of a Breast Cancer Specialized Program of Research Excellence grant and is designated the SPORE database. Steroid receptor assays for this database were done by identical methods at Nichols Institute (San Juan Capistrano, CA). Histologic diagnoses for both databases were made by pathologists at community hospitals. Patients who received adjuvant chemotherapy were excluded from the analyses because the focus of this article was to evaluate the role of PR methylation, both for prognosis in systemically untreated patients and for predicting response to tamoxifen therapy. The P01 database contains information about patients with early breast cancer who were diagnosed and treated between 1970 and 1998. Information about adjuvant therapy, disease recurrence, and death was obtained from physicians who were involved in the management of the breast cancer of patients. External validation against the Surveillance, Epidemiology and End Results Registry and other data sources indicates that this information has been reliably ascertained. The SPORE database contains patients with early breast cancer who were diagnosed and treated between 1970 and 1999 from hospitals throughout the United States. Follow-up information was obtained primarily from tumor registries. External validation indicates that death has been reliably ascertained; however, determination of first disease recurrence was often under-ascertained. The tumors have been stored in −70°C freezers at BCM since 1999, with an unintended interruption in 2001, when electricity was lost as a result of tropical storm Alison, and the samples thawed for 2 to 3 days. This however, should not affect studies with DNA, because DNA (and its modification such as methylation) is extremely stable.
PCR primers. The following PCR conditions were used: 0.1 mmol/L dNTPs, 1 U Taq polymerase, and 100 nmol/L forward and reverse primers. Two alternative translational initiation sites for PRA and PRB are shown as AUG2 and AUG1, respectively. The location of PRA and PRB promoters are shown at +464 to +1,105 and −711 to +31, respectively, as previously described (9). Two regions used for pyrosequencing assays are indicated by horizontal arrows. CpG islands are shown by striped boxes at the bottom of the figure.

Figure 1. Schematic of PRA and PRB promoters and location of primers. The positions of 2 alternative transcription start sites, PRA and PRB, respectively, are indicated by bent arrows. Two alternative translational initiation sites for PRA and PRB are shown as AUG2 and AUG1, respectively. The location of PRA and PRB promoters are shown at +464 to +1,105 and −711 to +31, respectively, as previously described (9). Two regions used for pyrosequencing assays are indicated by horizontal arrows. CpG islands are shown by striped boxes at the bottom of the figure.

Previous studies have found that the half-life of methylcytosine in double-stranded DNA at 37°C is approximately 30,000 years (19, 20). A number of quality control studies done by us, like PCR reactions yielding products of up to 1 kb, and analysis of highly methylated genes confirmed quality of DNA (data not shown).

DNA bank

From the 2 databases described above, we generated the BCM Breast Tumor DNA Bank-v1 by selecting 500 tumors from patients who did not receive adjuvant therapy after surgery ("untreated"), and 500 tumors from patients treated with tamoxifen ("tamoxifen treated"). Additional selection criteria were complete information about patient and tumor characteristics, and sufficient tumor material. A total of 213 samples came from the P01 and 787 from the SPORÉ banks. The median follow-up for patients still living is 122 and 124 months for untreated and tamoxifen-treated breast cancer patients, respectively. The amount of ER and PR in the tumor tissue was measured by ligand-binding assays as previously described (21). Tumors with an ER content of at least 3 fmol/mg protein (the limit of detection in this assay) and with a PR content of at least 5 fmol/mg protein were considered to be positive for ER and PR, respectively. DNA was isolated by using Puregene DNA Purification Kit (Qiagen) in the BCM Genetics Core, and eluted into 150 µL TE, pH = 8, yielding an average concentration of 525 ± 315 ng/µL (range 15–2,908 ng/µL).

Bisulfite pyrosequencing

One microgram of the isolated genomic DNA was bisulfite converted by using the EZ DNA Methylation Gold Kit (Zymo Research) according to the supplier’s protocol. Bisulfite pyrosequencing was done as previously described (22, 23). Briefly, bisulfite-treated DNA (40 ng) was amplified with gene-specific primers in a 2-step PCR. The second step of PCR was used to label single DNA strand with biotin by using a universal primer tag or gene-specific primers biotinylated at the 5' end. Each PCR step was done in a total volume of 20 µL of 67 mmol/L Tris-HCl (pH = 8.8), 16 mmol/L ammonium sulfate, 2 mmol/L MgCl₂, 0.125 mmol/L dNTPs, 1 U Taq polymerase, and 100 nmol/L PCR primers. The following PCR conditions were used: initial denaturation at 95°C for 5 minutes, followed by 40 cycles comprising denaturation at 94°C for 15 seconds, annealing at the appropriate temperature for 30 seconds, and extension at 72°C for 15 seconds. We used 45 cycles for the second step to completely exhaust the biotinylated primer. PCR primer sequences and annealing temperatures are listed in Supplementary Table S1. We determined levels of DNA methylation as the percentage of bisulfite-resistant cytosines at CpG sites by pyrosequencing with the PSQ HS 96 Pyrosequencing System (Biotage) and Pyro Gold CDT Reagents (Biotage), as previously described (22). Pyrosequencing assays interrogated 4 adjacent CpG sites for each PRA and PRB promoter. PRA and PRB promoter methylation has previously been associated with isoform-specific expression (24, 25). These reports suggest that hypermethylation can selectively silence PRA and PRB promoters in various cancer cell lines and tissues and that treatment with DNA methyltransferase inhibitors can restore expression, suggesting that inactivation is mediated through promoter methylation. PRB CpG sites are located at +150, +163, +166, +174, and PRA CpG sites are at +831, +839, +847, and +853 (the numbering is relative to PRB transcriptional start site +1, as originally described by Kastner and colleagues; ref. 3). The position of the primers within PRA and PRB genes are shown in Figure 1. The PRA CpG sites have been previously analyzed by others and were found to be associated with expression (25, 26). We found high concordance in methylation between adjacent sites and we therefore used mean values from all pyrosequenced CpG sites as a measure of methylation of a given gene. As previously described (27), methylation of 10% or above was used as a cutoff for presence of methylation.

Statistical analysis

The statistical methods were conducted in a multistage manner. First, descriptive and summary statistics were used to describe the patient and tumor characteristics in the dataset. Next, associations between clinical and molecular biomarkers were evaluated with scatter plots, Spearman rank correlations, linear regression, and 2 × 2 tables with the χ² test and Fisher’s exact test. Survival analysis was conducted separately for treated and untreated subjects to evaluate the effects of PR, PRA, and PRB on overall survival using the log-rank test and Kaplan-Meier survival curve.
survival (OS) and time to first recurrence (TTFR). OS is defined as the period of survival following surgery until death or loss-to-follow-up. TTFR is the period of recurrence-free survival following surgery until first recurrence or censoring, either because of death or loss-to-follow-up. Univariable and multivariable survival analyses were conducted by using Cox regression, and the proportional hazards assumption was checked in univariable models by using time-varying covariates (Methylation was studied as categorical and continuous variable). Kaplan–Meier curves and the log-rank test were also used for the survival analysis. All analyses were conducted by using SAS 9.2 and R software.

Results

Patient and tumor characteristics of cohort

The DNA for this cohort was isolated from breast tumors from 500 breast cancer patients treated with tamoxifen and 500 patients which did not receive endocrine treatment. In general, this is a very good outcome patient population in which tumors are characterized by small size (48% are smaller than 2 cm) and low rates of metastasis (66% are node negative; Table 1). All tumors were ER positive and 77% were PR positive. There was a positive correlation between ER levels and PR levels ($r = 0.34, P < 0.001$). Among the subjects that were younger than 50 years at diagnosis ($n = 109$), 33 were tamoxifen treated [PR+: $n = 30$ (90.9%), PR−: $n = 3$ (9.1%)] and 76 were untreated [PR+: $n = 62$ (51.6%), PR−: $n = 14$ (18.4%)]. Among the subjects that were 50 years or older at diagnosis ($n = 891$), 467 were tamoxifen treated [PR+: $n = 363$ (77.7%), PR−: $n = 104$ (22.3%)] and 424 were untreated [PR+: $n = 315$ (74.3%), PR−: $n = 109$ (25.7%)].

As expected, univariable survival analysis showed significant association between tumor size and TTFR in the tamoxifen-treated group ($P < 0.0001$) and in the untreated group ($P < 0.0001$). In the tamoxifen-treated patients, there was also an association between tumor size and OS ($P < 0.001$). Similarly, there was a significant association between nodal status and TTFR in both the tamoxifen-treated ($P < 0.0001$) and the untreated group ($P = 0.006$) and with OS in the tamoxifen-treated group ($P < 0.001$).

There is a significant difference in some of the patient and tumor characteristics, including age, tumor size, and nodal status, between patients who received tamoxifen treatment and those who did not (Table 1). This finding simply reflects the nature of the study, which is not a randomized prospective trial but a retrospective population-based analysis, and thus patients with more aggressive

<table>
<thead>
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<th>Characteristics of the study population and the treatment subsets</th>
<th>Tamoxifen treatment</th>
<th>$P$ (no vs. tam)</th>
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<tr>
<td>All</td>
<td>1,000 100</td>
<td></td>
</tr>
<tr>
<td>Age &lt; 50 y</td>
<td>100 15.2</td>
<td></td>
</tr>
<tr>
<td>≥ 50 y</td>
<td>100 93.4</td>
<td>&lt;0.001</td>
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<tr>
<td>ER Positive: ≥ 3 fmol/mg</td>
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<td>0.22</td>
</tr>
<tr>
<td>Negative: &lt; 5 fmol/mg</td>
<td>377 75.4</td>
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</tr>
<tr>
<td>Tumor size ≤ 2 cm</td>
<td>280 56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt; 2 and ≤ 5 cm</td>
<td>200 40</td>
<td></td>
</tr>
<tr>
<td>&gt; 5 cm</td>
<td>20 4</td>
<td></td>
</tr>
<tr>
<td>Nodes Node negative</td>
<td>405 81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1–3 nodes positive</td>
<td>68 13.6</td>
<td></td>
</tr>
<tr>
<td>4–9 nodes positive</td>
<td>20 4</td>
<td></td>
</tr>
<tr>
<td>10 or more positive nodes</td>
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</tr>
<tr>
<td>Relapse status No recurrence</td>
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<tr>
<td>Recurred</td>
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<td>Vital status Alive</td>
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<td>0.08</td>
</tr>
<tr>
<td>Deceased</td>
<td>179 35.8</td>
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Table 1. Patient and tumor characteristics

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tumors were more likely to receive additional adjuvant treatment, as decided by their treating physicians.

Given that the overall goal of our study was to evaluate PR methylation, we first determined the prognostic and predictive value of PR levels by using ligand-binding data. PR positivity was statistically significantly associated with better OS in tamoxifen-treated group (HR = 0.61, P = 0.002) and in the untreated group (HR = 0.54, P < 0.001; Fig. 2). PR positivity was also associated with improved TTFR in the untreated group (HR = 0.47, P = 0.002) but not in the tamoxifen-treated group (Supplementary Fig. S1). Thus, in our cohort, low PR levels were prognostic and, possibly, also predictive for worse outcome, although the latter needs to be interpreted with caution due to the inherent limitations of retrospective analyses. A subsequent analysis of clinical and biological tumor characteristics and PR levels revealed an association between low PR levels and increased tumor sizes (P = 0.0004) and increased nodal involvement (P = 0.0067) in the untreated patients, suggesting poor prognosis of low PR tumors (Supplementary Tables S2 and S3).

Methylation of PRA and PRB promoters in breast tumors

Of the 1,000 DNA samples, we successfully carried out bisulfite pyrosequencing assays for PRA and PRB on 993 and 997 tumors, respectively. The remaining samples were excluded because we were unable to obtain PCR products after repeated attempts. PR methylation was observed in a small subset of tumors—95 of 993 (9.5%), and 141 997 (14.1%) tumors were methylated in their PRA and PRB promoters, respectively (Fig. 3A). The overall distribution of methylation was similar for PRA and PRB as shown in Figure 3B.

In 178 tumors of the 1,000 tumors (17.8%), at least 1 promoter was methylated (Fig. 3C). There was a statistically significant association between PRA and PRB methylation (r = 0.71, P < 0.0001). However, only 39% of the variability of PRA was accounted for by PRB as described by the regression analysis (R² = 0.39). Indeed, there was a large subset of tumors in which isoform-specific methylation occurred. Specifically, we detected PRA and PRB-specific methylation in 39 (3.9%) and 83 (8.3%) tumors, respectively.

Association between PRA and PRB methylation with PR and ER expression

To determine whether PR methylation was associated with loss of PR expression, we compared PRA and PRB methylation with PR protein levels obtained from ligand-binding assay. As expected, the Spearman rank correlation revealed a significant inverse association between PR expression and PRA methylation (r = -0.38, P < 0.0001) and PRB methylation (r = -0.35, P < 0.0001). Although statistically highly significant, the biological associations were not as strong as one might have expected, given the general notion of promoter methylation resulting in loss of expression. Of the 227 PR-negative tumors (lacking expression of PRA and PRB), PRA was methylated...
in 59 tumors and unmethylated in 168 tumors (Fig. 4). Similarly, of the 228 PR-negative tumors (with information on PRB promoter methylation), PRB was methylated in 65 tumors and unmethylated in 163 tumors. These findings suggested that in the majority of PR-negative tumors, loss of PRA or PRB was not a result of DNA methylation in regulatory regions of the PR genes. On the other hand, one-third (n = 19; 34%) of PRA- and PRB-methylated tumors (n = 56) were classified as PR-positive tumors, suggesting that there is a subset of tumors in which PR methylation is not sufficient to completely silence PR expression.

Finally, we determined correlations between PR methylation and ER levels (although our study population consisted of exclusively ER-positive patients as determined by ligand-binding assay, the degree of ER levels in the tumors varied widely). There was a negative correlation between ER levels and PRA and PRB methylation (PRA: r = −0.16 and for PRB: r = −0.20, for both tests P < 0.001). ER and PR levels were highly correlated, as expected (r = 0.35, P < 0.001).

Association between PR methylation and outcomes
To examine whether PR methylation was associated with outcome in breast cancer patients, we conducted univariate Cox regression analysis by using the tamoxifen-treated and untreated patient cohorts. In the tamoxifen-treated group, OS was worse for patients with methylated PRA compared with unmethylated PRA (HR = 1.53; P = 0.04; Fig. 5A).

![Figure 4. Association between PR methylation and PR levels A, association of PRA, PRB, and both PRA and PRB methylation with PR levels from ligand-binding assay. B, Venn diagram representing tumors with PRA methylation, PRB methylation, and the overlap group (PRA and PRB methylation) in PR-negative and PR-positive breast tumors.]

![Figure 5. Association between PR isoform methylation and OS. The Y-axis indicates the percentage OS and the X-axis indicate the time from diagnosis to death in months. In all graphs 10% or above was used as a cutoff for presence of methylation. A, patients with no PRA methylation (n = 451; solid line) were compared with patients with PRA methylation (n = 47; dotted line) in the tamoxifen-treated group (left) and patients with no PRA methylation (n = 447; solid line) were compared with patients with PRA methylation (n = 48; dotted line) in the untreated group (right). B, patients with no PRB methylation (n = 425; solid line) were compared with patients with PRB methylation (n = 73; dotted line) in the tamoxifen-treated group (left) and patients with no PRB methylation (n = 431; solid line) were compared with patients with PRB methylation (n = 88; dotted line) in the untreated group (right). Short vertical lines indicate censored events. Abbreviation: n.s., not significant.]
Of note, the PRA-methylated tumors and the low PR tumors that showed worse OS are not the same tumors. Although there is some overlap between the low PR-expression tumors, and the PRA-methylated tumors, it is not complete because PRA-methylated tumors could either have low PR or high PR expression. Among tamoxifen-treated subjects, most of the PR-positive tumors are unmethylated [PRA unmethylated: \( n = 373 \) (95.2%); PRA methylation: \( n = 19 \) (4.9%)] and most of the PR-negative tumors are unmethylated [PRA unmethylated: \( n = 78 \) (73.6%); PRA methylation: \( n = 28 \) (26.4%)].

There was no significant difference in TTFR by PRA methylation in the tamoxifen-treated group (Supplementary Fig. S2A). In the untreated patients, subjects with PRA-methylated tumors did not have significantly worse OS than those with PRA nonmethylated tumors (although the PRA-methylated tumors trended toward worse OS; \( P = 0.09 \)). A subsequent analysis of clinical and biological tumor characteristics and PRA methylation revealed an association between PRA methylation and increased tumor sizes (\( P = 0.04 \)) and increased nodal involvement (\( P = 0.01 \)) in the untreated patients, supporting a general worse prognosis of these tumors (Supplementary Tables S4 and S5).

To evaluate the relationship between PRB methylation and survival, we conducted a similar analysis on patients treated with tamoxifen and patients who did not receive any systemic therapy. There was no significant effect of PRB methylation on OS (Fig. 5B) or TTFR (Supplementary Fig. S2B) in either group, suggesting that the observed association is specific. Similarly, we did not detect any association between PRB methylation and other clinical or biological characteristics of the tumors (Supplementary Tables S6 and S7).

Because in our study population, PR promoter methylation accounted for loss of PR in a small subset of PR—breast tumors, suggesting that other mechanisms were involved in silencing PR, we carried out further exploratory subgroup analysis stratifying these 2 groups. We did not find any statistically significant differences in outcome comparing groups with PR loss by methylation or PR loss by other mechanisms in tamoxifen-treated or untreated groups (data not shown). Furthermore, we did not detect any significant differences of the PR-methylated group (PRA, PRB, or both PRA and PRB methylation) with and without PR expression.

Finally, we asked the question whether the association between PRA methylation and outcome was driven by ER levels. In our study population, ER levels from the ligand-binding assay (as continuous variable) were not associated with OS in tamoxifen-treated subjects (HR = 1.00, \( P = 0.23 \)). ER levels were also not associated with OS in tamoxifen-treated subjects after accounting for PRA methylation (HR = 1.00, \( P = 0.18 \)). Therefore, the association between PRA methylation and poor outcome was an independent prediction, a critical and novel finding from our study.

Discussion

PR exists in 2 isoforms, PRA and PRB, which have distinct roles in regulating the effect of progesterone. PR levels in breast tumors were traditionally measured by ligand-binding assays and are currently assessed by immunohistochemistry. However, neither approach is able to discriminate between PRA and PRB. The expression of PR is epigenetically regulated and there is some evidence that PRA and PRB promoter methylation is associated with isoform-specific expression (24, 25). Therefore, the presence of DNA methylation at each promoter region could be potentially used as a surrogate marker for PR isoform status. In this study, we tested for association of PRA and PRB methylation with clinical outcome by using tumor DNA from breast cancer patients treated with adjuvant tamoxifen after surgery or surgery only. Our results provide the first direct demonstration that PR isoform-specific methylation impacts the clinical treatment response of breast cancer patients. In addition, we show that PR promoter methylation is associated with loss of PR in a small subset of PR-negative breast tumors, suggesting that other mechanisms are involved in silencing PR in the majority of PR-negative tumors.

PR levels and outcome

Low PR levels were associated with significantly worse OS in the untreated group, suggesting a prognostic significance of PR status. Consistent with our finding, earlier reports which have been validated through the years, suggested that patients with ER+/PR—breast cancers have a worse prognosis than patients who have ER+/PR+ tumors (28–30). In addition, we found that low PR levels were associated with worse OS in the tamoxifen-treated group. Given that our study is a retrospective cohort analysis, we cannot distinguish whether PR levels truly predict tamoxifen response or whether levels are mainly prognostic and independent of therapy. Nevertheless, preclinical and some clinical studies strongly suggest that absence of PR levels are associated with specific resistance to endocrine therapies like selective ER modulators, for example tamoxifen (31–36). However, the retrospective and nonrandomized nature of study design in most of these studies limits the ability to determine whether PR status has a true predictive significance. Therefore, suggestions that PR would serve as an important predictive marker for decision making regarding endocrine treatment in women with ER+ breast cancer remains controversial.

PRA and PRB methylation in breast tumors

In our study, PRA promoter was methylated in 9.5% of breast tumors and PRB promoter was methylated in 14.1% breast tumors, suggesting that PR promoter methylation is not a frequent event in breast cancer. Similar results were found by Feng and colleagues (27); however, Gaudet and colleagues reported that PRB (but not PRA) methylation was a frequent event (92.2%), although the actual levels of
methylation were weak (18). Furthermore, another group reports higher frequency of PRB methylation in sporadic breast tumors (26). It should be noted that inconsistency of reported methylation frequencies could be partly due to different sensitivities of methods used for measuring methylation, highlighting the need for standardized assays. For example, methylation-specific PCR (MSP) is a very sensitive method which can result in artificially high methylation rates, because of its ability to amplify methylation in a very small subset of the starting material and is, therefore, semiquantitative at best. Finally, it is important to note that our cohort was limited to ER-positive tumors; it is possible that loss of PR by methylation can be more frequently found in the more aggressive ER-negative tumors.

Although there was a statistically significant correlation between PRA and PRB methylation, with 56 tumors having both promoter methylated, there was substantial number of tumors which had only PRA (n = 39) or only PRB (n = 83) methylation. The occurrence of isoform-specific methylation events is supported by recent findings from Gaudet and colleagues, which did not find a significant correlation between PRA and PRB methylation in breast tumors (18). These findings support the idea that the expression of the 2 isoforms can be regulated by different signals, resulting in differential expression. Given the previously reported differences in downstream target genes, this finding highlights the need for differential measurement and discussion of PRA and PRB, instead of total "PR."

**Correlation of PRA and PRB methylation with PR and ER expression**

DNA methylation at each PR isoform promoter was significantly associated with reduced expression of total PR levels. Similar results were reported in a study of 200 breast tumors determining PRA and PRB methylation (18). Other studies, although not distinguishing PRA and PRB methylation, have also shown consistent results with respect to association between PR methylation and loss of expression (16, 37). However, reports by Widschwendter and others showed a lack of association between methylation of PR (in that case PRB) and PR status (38). It is important to note that in our study, despite the significant association of PR methylation with low PR levels, a substantial number of tumors in which both PRA and PRB isoforms were methylated, expressed PR protein. This finding suggests that very low or weak methylation levels in some tumors may be insufficient to completely inactivate the gene expression. Another explanation is simple tissue heterogeneity—it is possible that some tumors contained normal tissue which expressed PR at levels sufficient for detection by ligand-binding assays.

We also found that PR was not methylated in the majority of PR-negative tumors. Thus, although PR promoter methylation results in loss of PR expression, describes a subset of breast cancers, it is clearly not the sole or major mechanism of PR inactivation. Other epigenetic regulations, such as histone modifications, are likely to be involved, given that treatment with HDAC inhibitor induces re-expression of the PR gene in PR negative breast cancer cell lines (39–42). There is also increasing evidence that silencing of PR gene is associated with marked elevation in H3K27 tri-methylation levels, a process which is mediated by polycomb complexes 2 (43, 44). Importantly, prior studies have shown that kinase signaling pathways can alter PR expression at the transcriptional and posttranslational levels. Kim and colleagues have reported that PI3K/AKT/mTOR pathway activation resulted in decreased PR protein and mRNA levels in MCF-7 cells without altering ER levels or activity (45). Similarly, PTEN loss, which increased phosphoinositide 3-kinase (PI3K) pathway activation, was associated with decreased PR expression in a number of cell lines (46) and in human breast tumors (47).

In our study, there was a negative correlation between PR methylation and ER levels, with PR methylation being predictive for low ER levels. Similar observations of inverse associations between PR methylation and ER levels have previously been reported (18, 38). Because ER transcriptionally upregulates PR, methylation and absence of PR expression may have significant impact on breast cancer biology and response to endocrine therapy (see ref. 48 for review on PR levels and response to endocrine therapy). At this point, it is not clear how methylation of PR regulates loss of ER, or vice versa, how low ER levels could result in increased methylation of PR, but given the importance of ER expression, this deserves further and more detailed studies. Finally, there are a number of studies showing that the 5′ region of the ESR1 gene is frequently methylated in (ER−) breast tumors (17).

Future studies should address the question whether a subset of tumors with both ESR1 and PR methylation show a different biology and outcome compared with tumors in which only one of the receptor is methylated.

**Association between PRA methylation and outcomes in breast cancer patients**

A novel finding from our study is that methylation of PRA but not PRB is predictive for tamoxifen response. This finding is supported by some recent reports by using mouse mammary carcinoma models in which loss of PRA was associated with endocrine resistance. Briefly, Wargon and colleagues have developed 3 lines of antiprogestin-resistant mouse mammary carcinoma by prolonged exposure to RU-486 (15). Consistently, in all lines, PRA expression was decreased, with little changes in PRB, suggesting that loss of PRA is associated with hormone resistance in this model. The authors subsequently showed that PRA was methylated in the resistant tumors and that treatment with methylation inhibitors can reverse resistance and results in re-expression of PRA (49).
There is one prior study which addressed the role of PR isoforms in tamoxifen resistance (14). In this article, in which PR expression was measured by immunoblotting, lack of treatment response was associated with an increased PRA/PRB ratio. If one assumes that loss of PRA protein expression would be associated with PRA methylation, then these results are somewhat inconsistent with ours. There are many reasons for these differences, including details of tumor specimens, and methods used for the study. There is some discussion that quantitative measurement of PR isoforms by immunoblotting is complicated by the fact of a lower band, which does not separate on high percentage SDS gels, which could potentially perturb the measurement of an accurate PRA/PRB ratio (15).

In any case, these findings strongly suggest the critical need for a better understanding of PR isoform-specific functions and expression in breast tumors. There is increasing realization that PRA and PRB play different roles in modulating cellular response to progesterone by differentially regulating downstream gene expression (50). PRA methylation might contribute to tamoxifen resistance by altering the PRA:PRB ratio so that PRB is predominant PR isoform. PRB is known to be a stronger transcriptional activator than PRA and it may upregulate genes known to be involved in tumors aggressiveness and poor prognosis. PRB was also shown to affect ER–estrogen response element (ERE) interaction and cellular uptake of estrogen in a ligand-independent manner (51). Finally, the Horwitz group showed that PRA has transrepression activity, that is, it can suppress the transcriptional activity of PRB and of other nuclear receptors, including ER. Thus, loss of PRA resulting from hypermethylation could cause increased PRB and ER activity and thereby contributing to endocrine resistance (52).

Given the increasing literature on a critical role of progesterone in breast cancer and differential expression and function of PRA and PRB, more studies are needed to unravel the role of the PR isoforms in breast cancer development and endocrine treatment response. Such knowledge is also important for the development of PR-targeted drugs, which should be isoform and tissue specific.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Progesterone Receptor Isoform-Specific Promoter Methylation: Association of PRA Promoter Methylation with Worse Outcome in Breast Cancer Patients

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