Progesterone receptor isoform-specific promoter methylation — Association of PRA promoter methylation with worse outcome in breast cancer patients.

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Statement of Translational Relevance.

Our analysis of PRA and PRB promoter methylation provides two 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. This data suggests 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 to ER-positive/PR-positive tumors, it is critical to decipher those mechanisms in more detail in order to identify novel treatment targets.
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 study 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 has is frequently used as surrogate marker for functional ERα activity. PR exists in two isoforms, PRA and PRB, which are the result of transcription from two alternative promoters, and initiation of translation at two different AUG codons (3). Structurally, PRB differs from PRA only in that the B 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 two 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, while glands from PRB transgenic mice have inappropriate lobulo-alveolar 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 co-expressed 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 et al recently showed that acquired antiprogestin-resistant mammary gland tumors in mice have low levels of PRA, and suggested that high levels of PRA expression might be a marker of antiprogestin responsiveness (15).

It has been more than a decade ago when it was originally shown that loss of PR expression was associated with promoter methylation (16). Since then, a number of studies have analyzed PR promoter methylation in breast tumors (reviewed in (17)), with limited consensus on rates of methylation, which is at least in part is due to the use of different methods. Also, many studies were based on small sets of clinical samples from heterogeneously treated patients without limited annotation or selection criteria. Moreover, only one recently published study has analyzed, compared and contrasted methylation of PRA
and PRB promoter in breast cancers (18). And finally, to our knowledge, none of the studies have addressed association between PR isoform-specific methylation and endocrine treatment response.

Therefore, we performed a large population-based study to test for association of PRA and PRB methylation with PR expression, and with clinical outcome by using tumor DNA from 500 breast cancer patients treated with adjuvant tamoxifen after surgery, and 500 patients systemically untreated. Here we show that low PR expression is significantly associated with worse outcome in the tamoxifen treated group and the surgery only group. Methylation of PRA and PRB is inversely associated with PR expression; however, the majority of PR-negative tumors are not methylated. While PRA methylation is significantly associated with PRB methylation, there is a subset of tumors in which PRA only or PRB only was methylated, suggesting independent regulation of PRA and PRB methylation. Intriguingly, methylation of PRA, but not PRB was significantly associated with worse outcome in tamoxifen treated breast cancer patients.
**Materials and Methods**

**Study population**

The Breast Center at BCM (Houston, TX) maintains two 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 NCI (Bethesda, MD) as part of a Program Project grant and is designated the PPG/P01 database. All receptor assays were performed 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 performed 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 study 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 patients’ breast cancer. 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 US. 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 2-3 days. This however, should not affect studies with DNA, since DNA (and its modification such as methylation) is extremely stable. Previous studies have found that the half-life of methyl-cytosine in double-stranded DNA at 37°C is approx 30,000 years (19, 20). A number of quality control studies performed by us, such as PCR reactions yielding products of up to 1kb, and analysis of highly methylated genes confirmed quality of DNA (data not shown).

**DNA Bank**

From the two 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. 213 samples came from the P01, and 787 from the SPORE 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 using Puregene® DNA Purification Kit (Quiagen) in the BCM Genetics Core, and eluted into 150 ul TE pH 8, yielding an average concentration of $525 \pm 315$ ng/ul (range 15-2908 ng/ul).

Bisulfite pyrosequencing

One ug of the isolated genomic DNA was bisulfite converted using the EZ DNA methylation gold kit (Zymo Research) according to the supplier’s protocol. Bisulfite pyrosequencing was performed as previously described (22, 23). Briefly, bisulfite-treated DNA (40 ng) was amplified with gene-specific primers in a 2-step polymerase chain reaction (PCR). The second step of PCR was used to label single DNA strand with biotin using a universal primer tag or gene-specific primers biotinylated at the 5' end. Each PCR step was performed in a total volume of 20 µL of 67 mM Tris-HCl (pH = 8.8), 16 mM ammonium sulfate, 2 mM MgCl2, 0.125 mM dNTPs, 1 U Taq polymerase, and 100 nM 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 Supplemental Table 1. 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, Charlottesville, VA) 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 et al (3)). The position of the primers within PRA and PRB genes are shown in Figure 1. The PRB 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 cut off for presence of methylation.

**Statistical Analysis**

The statistical methods were conducted in a multi-stage 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 scatterplots, Spearman rank correlations, linear regression, and 2x2 tables with the chi-square ($\chi^2$) 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 and time to first recurrence. Overall survival (OS) is defined as the period of survival following surgery until death or loss-to-follow-up. Time to first recurrence (TTFR) is the period of recurrence-free survival following surgery until first recurrence or censoring, either due to death or loss-to-follow-up. Univariable and multivariable survival analyses were conducted using Cox regression, and the proportional hazards assumption was checked in univariable models 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 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 < 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 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 time to first recurrence (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 overall survival (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 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 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) (Figure 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 figure 1). 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 2 and 3).

*Methylation of PRA and PRB promoters in breast tumors*

Of the 1000 DNA samples, we successfully performed bisulfite pyrosequencing assays for *PRA* and *PRB* on 993 and 997 tumors, respectively. The remaining samples were excluded since we were unable to obtain PCR products after repeated attempts. PR methylation was observed in a small subset of tumors — 95 out of 993 (9.5%), and 141 out of 997 (14.1%) tumors were methylated in their *PRA* and *PRB* promoters respectively (Figure 3A). The overall distribution of methylation was similar for *PRA* and *PRB* as shown in Figure 3B.

In 178 tumors of the 1000 tumors (17.8%), at least one promoter was methylated (Figure 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 if 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. Out of 227 PR-negative tumors (lacking expression of *PRA* and *PRB*), *PRA* was methylated in 59 tumors, and unmethylated in 168 tumors (Figure 4). Similarly, out of 228 PR-negative tumors (with information on *PRB* promoter methylation), *PRB* was methylated in 65 tumors, and unmethylated in 163 tumors. These findings suggest 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 consists 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 if \( PR \) methylation was associated with outcome in breast cancer patients, we performed univariate Cox regression analysis using the tamoxifen treated and untreated patient cohorts. In the tamoxifen treated group, OS was worse for patients with methylated \( PRA \) compared with non-methylated \( PRA \) (HR=1.53; \( p=0.04 \)) (Figure 5A).

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, since \( PRA \) methylated tumors could be 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 Figure 2A). In the untreated patients, subjects with \( PRA \) methylated-tumors did not have significantly worse OS than those with \( PRA \) non-methylated 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 4 and 5).

To evaluate the relationship between \( PRB \) methylation and survival, we performed 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 (Figure 5B) or TTFR (Supplementary Figure 2B) in either group suggesting that the observed association is \( PRA \) specific. Similarly, we did not detect any association between \( PRB \) methylation and other clinical or biological characteristics of the tumors (Supplementary Tables 6 and 7).

Since in our study population, \( PR \) promoter methylation accounts for loss of PR in a small subset of PR- breast tumors, suggesting that other mechanisms are involved in silencing PR, we performed further exploratory subgroup analysis stratifying these two 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). Further, 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 overall survival in tamoxifen-treated subjects (HR=1.00, p=0.23). ER levels were also not associated with overall survival in tamoxifen-treated subjects after accounting for *PRA* methylation (HR=1.00, p=0.18). Therefore, the association between *PRA* methylation and poor outcome is an independent prediction, a critical and novel finding from our study.
Discussion

PR exists in two 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 the present 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 such as selective ER modulators, for example tamoxifen (31-36). However, the retrospective and non-randomized nature of study design in most of these studies limits the ability to determine if 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 et al (27); however, Gaudet et al 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, due to its ability to amplify methylation in a very small subset of the starting material, and is therefore semi-quantitative 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 et al, 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 two 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, while 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- 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 (PRC) 2 (43, 44). Importantly, prior studies have shown that kinase signaling pathways can alter PR expression at the transcriptional and post-translational levels. Kim et al. 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 PI3K pathway activation, was associated with decreased PR expression in a number of cell lines (46), and in human breast tumors (47). Lange et al. have shown that MAPK-mediated phosphorylation of PRA and PRB promotes their degradation (47). Therefore, it will be of interest to further decipher the different mechanisms leading to loss of PR, and to determine whether there are differences in the biology of these tumors.

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). Since ER transcriptionally upregulates PR, methylation and absence of PR expression may have significant impact on breast cancer biology and response to endocrine therapy (see (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 to 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 using mouse mammary carcinoma models in which loss of PRA was associated with endocrine resistance. Briefly, Wargon et al have developed three 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 \textit{PRA} (49).

There is one prior study which addressed the role of PR isoforms in tamoxifen resistance (14). In this study, 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 \textit{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). \textit{PRA} methylation might contribute to tamoxifen resistance by altering the PRA: PRB ratio so that PRB is predominately PR isoform. PRB is known to be a stronger transcriptional activator than PRA and it may up-regulate 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 trans-repression activity, i.e. 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.
References:


Acknowledgements:
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Figure 1: Schematic of PRA and PRB promoters, and location of primers

The positions of two 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 +1105 and -711 to +31 respectively as previously described (3). 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 2: Association between PR expression and overall survival (OS)

Kaplan-Meier estimates of overall survival (OS) are shown for tamoxifen treated (n=500) (left panel) and untreated (n=500) (right panel) breast cancer patients. The y-axis indicates the percentage OS and the x-axis indicate the time from diagnosis to death in months. Patients with low/absent PR expression (<5fmol/mg) (solid line) were compared with patients with high PR expression (>=5fmol/mg) (dotted line). Short vertical lines indicate censored events.

Figure 3: PRA and PRB methylation levels in breast tumors

A) Methylation of both PRA and PRB promoters as measured by bisulfite pyrosequencing in 993 and 997 breast tumors respectively. B) Histogram showing the distribution of PRA and PRB methylation levels in breast tumors. C) Venn diagram representing tumors with PRA methylation, PRB methylation, and the overlap group (PRA and PRB methylation).

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 overall survival (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 cut off 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 panel) 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 panel). 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 panel) and patients with no PRB methylation (n=431) (solid line) were compared with patients with PRB methylation (n=68) (dotted line) in the untreated group (right panel). Short vertical lines indicate censored events.
Supplementary Figure 1: Association between PR expression and time to first recurrence (TTFR)

Kaplan-Meier estimates of time to first recurrence (TTFR) are shown for tamoxifen treated (n=500) (left panel) and untreated (n=500) (right panel) breast cancer patients. The y-axis indicates the proportion without first recurrence and the x-axis indicate the time in months. Patients with low/absent PR expression (<5fmol/mg) (solid line) were compared with patients with high PR expression (>=5fmol/mg) (dotted line). Short vertical lines indicate censored events.

Supplementary Figure 2: Association between PR isoform methylation and time to first recurrence (TTFR)

The y-axis indicates the proportion without the first recurrence and the x-axis indicates the time in months. In all graphs 10% or above was used as a cut off 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 panel) 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 panel). 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 panel) and patients with no PRB methylation (n=431) (solid line) were compared with patients with PRB methylation (n=68) (dotted line) in the untreated group (right panel). Short vertical lines indicate censored events.
**Figure 1**

A diagram showing the genomic regions and transcription start sites (TSS) for Promoter B and Promoter A. The figure includes the location of exons and the primers used for PCR. CpG Islands are indicated.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Tamoxifen treatment</th>
<th>All</th>
<th>P (No vs Tam)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>All</td>
<td>500</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Age &lt;50yrs</td>
<td>76</td>
<td>15.2</td>
<td>33</td>
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<tr>
<td>Age &gt;= 50yrs</td>
<td>424</td>
<td>84.8</td>
<td>467</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive: &gt;=3fmol/mg</td>
<td>123</td>
<td>24.6</td>
<td>107</td>
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<tr>
<td>Negative: &lt;5fmol/mg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive: &gt;=5fmol/mg</td>
<td>377</td>
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<td>393</td>
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<tr>
<td>&lt;=2cm</td>
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<td>200</td>
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<td>&gt;2 and &lt;=5cm</td>
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<td>40</td>
<td>273</td>
</tr>
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<td>&gt;5cm</td>
<td>20</td>
<td>4</td>
<td>27</td>
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<tr>
<td>Nodes</td>
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<tr>
<td>Node negative</td>
<td>405</td>
<td>81</td>
<td>258</td>
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<tr>
<td>1-3 nodes positive</td>
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<td>168</td>
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<tr>
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<tr>
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<td>1.4</td>
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<tr>
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<td>64.2</td>
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<tr>
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<td>179</td>
<td>35.8</td>
<td>206</td>
</tr>
</tbody>
</table>
Figure 2

Tamoxifen-treated

No adjuvant treatment

p=0.002

p<0.001

Proportion of OS vs Time in months
A) | $PRA$ (N=993) | Methylated | 95 (9.5%) |
| | Unmethylated | 898 (90.5%) |
| $PRB$ (N=997) | Methylated | 141 (14.1%) |
| | Unmethylated | 856 (85.9%) |

B) ![Graph showing % PRA and PRB methylation](image)

C) ![Circle diagram showing PRA, PRA + PRB, and PRB with % methylation](image)

**Figure 3**
A)  

<table>
<thead>
<tr>
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<th>PRA methylation</th>
<th>PRB methylation</th>
<th>PRA + PRB methylation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=95)</td>
<td>No (n=898)</td>
<td></td>
</tr>
<tr>
<td>PR- (n=227)</td>
<td>59 (26.0%)</td>
<td>168 (74.0%)</td>
<td></td>
</tr>
<tr>
<td>PR+ (n=766)</td>
<td>36 (4.7%)</td>
<td>730 (95.3%)</td>
<td></td>
</tr>
</tbody>
</table>

**p<0.0001**

B)  

![Figure 4](https://via.placeholder.com/150)

**Figure 4**
Figure 5
Clinical Cancer Research

Progesterone receptor isoform-specific promoter methylation - Association of PRA methylation with worse outcome in breast cancer patients.

Thushangi N Pathiraja, Priya B Shetty, Jaroslav Jelinek, et al.

Clin Cancer Res  Published OnlineFirst April 1, 2011.

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