DNA Hypermethylation of *ESR1* and *PGR* in Breast Cancer: Pathologic and Epidemiologic Associations

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Abstract

Improved understanding of the etiology of estrogen receptor-α (ERα)–negative and progesterone receptor (PR)–negative breast cancers may permit improved risk prediction. *In vitro* studies implicate DNA hypermethylation of the ERα and PR promoters in the pathogenesis of ERα-negative and PR-negative tumors, but results are not definitive. We evaluated 200 invasive breast cancers selected from a population-based case-control study. DNA extracted from fixed tumor tissue cores was tested using MethyLight to assess DNA methylation at four CpG islands: *ESR1* promoters A and B; *PGR* promoters A and B; and a CpG shore, *ESR1* promoter C. DNA methylation results were compared with levels of ERα and PR, tumor characteristics, and breast cancer risk factors. We observed mild to moderate DNA methylation levels in most tumors for *ESR1* promoters A and B and *PGR* promoter B, and a few tumors showed mild methylation in *PGR* promoter A. In contrast, *ESR1* promoter C showed a wide range of methylation and was weakly correlated with lower expression levels of ERα ($\beta = -0.26$; $P < 0.0001$) and PR ($\beta = -0.25$; $P < 0.0001$). The percentage of tumors with methylated *PGR* promoters A and B was significantly higher for tumors with low ERα (A, Fisher's test $P = 0.0001$; B, $P = 0.033$) and PR levels (A, $P = 0.0006$; B, $P = 0.001$). Our data suggest that the relationships between DNA methylation of *ESR1* and *PGR* promoters and protein expression are weak and unlikely to represent a predominant mechanism of receptor silencing. In contrast to CpG islands, *ESR1* promoter C showed a wider range of methylation levels and inverse associations with ERα and PR expression. (Cancer Epidemiol Biomarkers Prev 2009;18(11):3036–43)

Introduction

In the United States, the majority of breast cancers are diagnosed among postmenopausal women and express estrogen and progesterone receptors (i.e., ERα positive, PR positive; refs. 1-3). In contrast to ERα-negative tumors, studies of ERα-positive tumors have identified etiologic associations that suggest potential strategies for risk reduction (1, 3-5). Specifically, controlling postmenopausal obesity, limiting the use of hormone replacement medications, and chemoprevention with selective ER modulators represent promising avenues for reducing risk for ERα-positive tumors, but would have minimal impact on ERα-negative cancers (5). Accordingly, efforts to increase our knowledge about the pathogenesis of ERα-negative tumors are important, especially because these tumors disproportionately affect young women and African American women (6).

Among the mechanisms that have been linked to the development of receptor negative tumors, DNA hypermethylation of CpG islands in promoters of the ERα and PR genes has received the greatest attention (7). In experimental systems, DNA hypermethylation of gene promoter regions represents a somatically heritable mechanism for expression silencing (8). In particular, studies of some, but not all, breast cancer cell lines have shown that methylation is associated with decreased expression of ERα mRNA and protein (9-12), and that treatment with demethylating agents results in restoration of expression (13). However, translating these findings to human tumors has proved to be more complex. Although several studies have found statistically significant associations between the frequency of DNA promoter methylation and ERα status (14-16), data generally suggest that methylation is not restricted to ERα-negative tumors (12, 15) and some studies have found null associations between methylation and protein expression (17).

Most studies of the relationship between DNA methylation and gene expression of ERα and PR have been based on small sets of clinical samples without detailed annotation or selection criteria (7, 12-20). Analyses of large, representative samples of breast cancer cases are

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important to more fully define the relationships between DNA methylation and hormone receptor expression, tumor characteristics, and risk factors. Accordingly, we performed an analysis of 200 breast cancer cases ascertained in a population-based case-control study to examine the relationship between DNA methylation of *ESR1* and *PGR* and these factors.

Materials and Methods

Study Population. Cases were identified in a large population-based case-control study, conducted among women ages 20 to 74 y, residing in two Polish cities, Warsaw and Lodz, from 2000 to 2003 as described in detail elsewhere (21). Eligible cases were women diagnosed with incident histologically confirmed *in situ* or invasive breast cancer identified through a rapid identification system. Controls included women randomly identified in population lists who had not been diagnosed with breast cancer, frequency matched to cases by city and age. Institutional Review Board approval was obtained from all participating institutions, and signed informed consent was obtained for all respondents. A total of 2,386 cases (79% of the 3,037 eligible cases identified) and 2,502 controls (69% of the 3,639 eligible controls identified) were enrolled.

Enrollment Procedures. Trained interviewers queried participants about known or suspected breast cancer risk factors (21). Women were considered premenopausal if still menstruating at the time of interview, postmenopausal if periods had stopped, and unclear menopausal status if menopausal hormone replacement therapy was initiated before cessation of natural periods. Body mass index (BMI) was calculated using measured (during the time of the interview) weight (kg) divided by standing height (m) squared. For about 5% of subjects with missing information, BMI was calculated from self-reported information. Women who reported having had a benign breast biopsy 1 y before cancer diagnosis (cases) or interview (controls) were considered to have had a history of benign breast disease.

Pathology. Histopathologic features including histologic type, grade, tumor size, and axillary lymph node metastases were assessed using clinical reports and independent evaluation by the study pathologist (M.E.S.). Results of ERα and PR assays (negative versus positive) done for clinical management primarily using immunohistochemical stains of whole sections were available for 77% of cases.

Routinely prepared formalin-fixed, paraffin-embedded tissue blocks from 900 invasive breast cancers were used to construct five tissue microarray (TMA) blocks with 3-fold representation as 0.6-mm-diameter cores (Beecher Instruments). Tissue sections of 5-μm thickness were placed on glass slides using a tape-transfer system (Instrumedics, Inc.) with UV cross-linking, dipped in paraffin, and stored at room temperature under nitrogen to help preserve antigenicity. TMAs were analyzed for ERα and PR using Automated Quantitative Analysis (AQUA), an automated quantitative immunofluorescent assay (22, 23), as previously described (24). In an earlier set of TMAs from the study, ERα and PR expressions, as measured independently by AQUA and by visual assessment of conventional immunostains, were highly correlated ($r^2 > 0.80$). In addition, AQUA scores were significantly higher for cases with positive clinical tests for ERα and PR as compared with those that were negative (both comparisons, $P < 0.0001$; ref. 24). The 200 cases studied herein were represented in a new set of TMAs. One case had a missing AQUA value for expression of PR.

Tumor Sampling Pilot Study for MethyLight Analysis. To determine the number of tissue cores required to determine DNA methylation status, we conducted a pilot study of 12 tumors. First, we examined H&E-stained sections of the tumors to identify corresponding areas of essentially pure cancer in each block. Then, using a 1.0-mm-diameter TMA needle, we removed three cores of tissue scattered within these regions for testing. In between tumor core sampling, the TMA needle was "cleaned" by punching an empty paraffin block three times. DNA extracted from each fixed core (see "DNA Extraction Protocol") was quantified and assessed using a DNA ladder. Cores yielded DNA of sufficient quantity for MethyLight assays, consisting mainly of 500- to 1500-bp fragments. DNA was bisulfite modified and used to perform MethyLight analysis for *ESR1* and *PGR* promoter regions (see "Bisulfite Conversion and DNA Methylation Analysis"). The DNA methylation results expressed as percentage of methylated reference (PMR) relative to a standard of 100% methylated DNA were assessed categorically (0, >0-5, >5-50, >50) for five DNA methylation markers related to *ESR1* and *PGR*. The percentage of the 12 tumors that gave exact agreement between the three cores for the five loci tested ranged from 60% to 80%. Cases never had more than one discordant core, which always varied by one category from the other two (data not shown). Therefore, we concluded that one core was adequate for testing.

MethyLight Analysis at Five Loci Related to *ESR1* and *PGR* of Breast Cancer Cases. We randomly selected 200 eligible cases who were not taking postmenopausal hormones at baseline and who previously were included in a TMA (see "Pathology"). Single tumor-enriched cores (removed as noted above) were used for DNA extraction, bisulfite treatment, and MethyLight assays (see "Bisulfite Conversion and DNA Methylation Analysis"). We randomly tested 10 empty cores prepared to clean the TMA needle between core sampling for the presence of DNA; all were negative. Two cores were extracted for 20 cases to provide duplicate samples for MethyLight analysis.

DNA Extraction from Tissue Cores. Tumor-enriched DNA cores were deparaffinized by incubation in 1.0 mL of xylene at room temperature for 30 min, removing the fluid after centrifugation, and then repeating the deparaffinization procedure. The resulting DNA was then treated twice with two rinses of 0.5 mL of 100% ethanol and air-dried. We extracted DNA from the tissues by incubating the tubes overnight at 50°C in 50 μL of lysis buffer (100 mmol/L Tris, 1 mmol/L EDTA, 0.05% SDS, 1.5 mg/mL proteinase K, 0.05 mg/mL tRNA), followed by inactivation of proteinase K by heating for 8 min at 95°C.

Bisulfite Conversion and DNA Methylation Analysis. DNA-containing tissue lysates were subjected to bisulfite modification using the EZ DNA Methylation Kit (Zymo Research) following the protocol suggested by the manufacturer. After bisulfite conversion, the quantity of DNA to be used for MethyLight analysis was assessed

by testing a small aliquot of the recovered bisulfite-converted DNA by real-time PCR using an ALU-based, bisulfite-specific, methylation-independent control reaction (see details about this reaction in Supplementary Table S1). MethyLight reactions were designed for three of the multiple alternative promoters of the *ESR1* gene (promoters A, B, and C; ref. 25) and the two *PGR* alternative promoters (promoters A and B; ref. 26; Supplementary Fig. S1). Except for the CpG shore (27) near *ESR1* promoter C, all the other promoters were located in CpG islands (28, 29). The primer/probe sequences for these reactions are detailed in the Supplementary Table S1. MethyLight analyses for the five *ESR1* and *PGR* isoforms gene promoters were done as previously described (30, 31). MethyLight results measure the level of DNA methylation at specific loci in test samples compared with a completely methylated DNA reference, yielding PMR for specific CpGs of target genes (32).

Statistical Analysis. PMR values were assessed in two ways: as continuous log-transformed values approximating a normal distribution and as categorical measures. For consistency across publications using MethyLight, we used the categories and description of PMR values as previously defined (32, 33): no methylation (0 PMR); "mild" (>0-5 PMR), "moderate" (5-50 PMR), and "heavy methylation" (>50 PMR). ERα and PR expressions were measured based on AQUA scores. AQUA values for each protein were centered on the mean and then log-transformed. In statistical models, AQUA values were treated as continuous measures and categorized into positive (higher levels) and negative (lower levels) expression based on the distribution (separately by study site) of previous findings (34). The following breast cancer risk factors were treated as continuous variables when possible: education, parity, age at first birth (per 5-y increase), age at menarche, age at menopause (per 5-y increase), menopausal status, premenopausal and postmenopausal BMI (per 5-unit increase), family history of breast cancer, and history of benign breast disease.

To assess whether the cases selected for analysis were representative of all Polish cancers, we compared the distribution of subject characteristics for the subset of 200 subjects to that of all other cases using χ^2 tests. To assess reproducibility, we compared DNA methylation PMR values for duplicate cores of 20 samples using the κ statistic for categorical variables and Spearman correlation for continuous variables. Relationships between categorical variables were assessed using Fisher's exact test. Spearman correlation coefficients were calculated to estimate the association between continuous exposure and outcome variables. To assess determinants of DNA methylation, we used linear regression models with continuous PMR values as the outcome and continuous AQUA values and risk factor data as the explanatory variables. Age and study site were included in crude models, and multivariable models also included all risk factors under study. Associations between protein expression of ERα (categorized) and DNA methylation (categorized) of PR and vice versa were assessed using the Cochran Mantel-Haenszel test. To investigate whether the relationship between ERα expression and DNA methylation varied by PR status, we evaluated linear regression models that included a cross-product term of PMR values (continuous) and ERα expression (continuous).

Results

Study Population. The 200 cases selected for this study were similar to the larger population-based sample of cases with respect to most, but not all, epidemiologic risk factors (Supplementary Table S2). Cases in the present study were more likely to be from Lodz than in the complete study population. The tumors of cases in the present study were more likely to be poorly differentiated and have multiple nodal metastases (Supplementary Table S2), which was explained by differences in the characteristics of available tumor blocks in Lodz compared with Warsaw (data not shown). Of the 200 cases in the present analysis, 55 (27.5%) had chemotherapy, whereas none had radiotherapy or hormone therapy, before surgery to remove the tumor.

Univariate Distribution of PMR Values. Six of the 200 cases were excluded because of low DNA quantity as assessed by high ALU C(t) values (>21). DNA methylation (PMRs >0) was evident for 84.7% of breast tumors at *ESR1* promoter A, 98.0% at *ESR1* promoter B, 100% at *ESR1* promoter C, 7.7% at *PGR* promoter A, and 92.3% at *PGR* promoter B. The range of PMR values (0-31) was limited for each CpG region with the exception of *ESR1* promoter C, which ranged from 1.8% to 362.6% with only one sample exceeding 100%. The outlier was not removed from the analysis because further examination of the DNA quantity [as measured by ALU C(t)] and assay performance did not suggest a measurement artifact and may be an indicator of multiple copies of the gene. Given the range of values, *ESR1* promoter C PMR values were treated as both categorical and continuous variables. Correlation between 20 duplicate DNA methylation measurements for *ESR1* promoter C was strong ($r^2 = 0.60$, $P = 0.081$). Given the more limited range of the remaining CpG regions, the PMR values at these sites were categorized. Agreement between duplicate scoring of PMR values was 95% for *ESR1* promoter A, 85% for *ESR1* promoter B, 90% for *PGR* promoter A, and 75% for *PGR* promoter B. Greater DNA methylation was weakly significantly correlated at most CpG regions in *ESR1* and *PGR*; however, DNA methylation at *ESR1* promoter A was not correlated with DNA methylation at *PGR* promoter A or *PGR* promoter B, and DNA methylation at two *PGR* promoter regions was not correlated (Supplementary Table S3). Medians and categories of PMR values for the five CpG regions did not differ among tumors that received chemotherapy before surgery compared with tumors that did not receive treatment before surgery ($P > 0.25$ for the nonparametric equality of means and χ^2 tests, respectively; data not shown).

Categorical Analysis of DNA Methylation at *ESR1* and *PGR* Promoters and Expression of ERα. DNA methylation at *ESR1* promoter A was unrelated to protein expression (Table 1), whereas DNA methylation at promoters B and C showed borderline associations with lower ERα protein expression (for B, $P = 0.055$; for C, $P = 0.068$; Table 1). In contrast, DNA methylation at *PGR* promoters was more strongly related to lower ERα levels. Specifically, statistically significant relationships were found for lower ERα levels with DNA methylation at *PGR* promoter A ($P = 0.00012$) and promoter B ($P = 0.033$), although these data were driven by relatively small

Table 1. Relationships between DNA methylation PMR values and AQUA ERα protein expression (200 breast cancer cases from the Polish Breast Cancer Study, 2000-2003)

Promoters/PMR values	Low ERα expression (n = 74)	High ERα expression (n = 120)	P*
	n (%)	n (%)	
ESR1 promoter A			
0	10 (13.5)	18 (15.0)	
>0-5	64 (86.5)	102 (85.0)	
>5-50	0 (0.0)	0 (0.0)	
>50	0 (0.0)	0 (0.0)	0.84
ESR1 promoter B			
0	0 (0.0)	4 (3.3)	
>0-5	54 (73.0)	98 (81.7)	
>5-50	20 (27.0)	18 (15.0)	
>50	0 (0.0)	0 (0.0)	0.055
ESR1 promoter C			
0	0 (0.0)	0 (0.0)	
>0-5	2 (2.7)	7 (5.8)	
>5-50	56 (75.7)	101 (84.2)	
>50	16 (21.6)	12 (10.0)	0.068
PGR promoter A			
0	61 (82.4)	118 (98.3)	
>0-5	10 (13.5)	2 (1.7)	
>5-50	3 (4.1)	0 (0.0)	
>50	0 (0.0)	0 (0.0)	0.00012
PGR promoter B			
0	2 (2.7)	13 (10.8)	
>0-5	69 (93.2)	106 (88.3)	
>5-50	3 (4.1)	1 (0.8)	
>50	0 (0.0)	0 (0.0)	0.033

NOTE: MethyLight results measure the level of DNA methylation at specific loci in test samples compared with a completely methylated DNA reference, yielding PMR for specific CpGs of target genes (32). PMR values were categorized as previously defined (32): no methylation (0 PMR), mild (>0-5 PMR), moderate (5-50 PMR), and heavy methylation (>50 PMR). TMAs of breast tumors were analyzed for ERα, PR, and HER-2/neu using AQUA, an automated quantitative immunofluorescent assay (22, 23), as previously described and categorized in a previous Polish Breast Cancer Study publication (24).
*Fisher's exact test.

differences in the number of cases per DNA methylation category. Of 15 cancers without detectable DNA methylation at *PGR* promoter B, 13 (87%) showed high ERα expression, whereas only 72 of 179 (40%) with detectable DNA methylation had high levels (Table 1).

Categorical Analysis of DNA Methylation at *ESR1* and *PGR* Promoters and Protein Expression of PR. DNA methylation at *ESR1* promoters was not significantly related to expression of PR (Table 2). However, DNA methylation at *PGR* promoter A (P = 0.00057) and that at promoter B (P = 0.0013) were related to low levels of PR protein. Of 15 tumors without detectable DNA methylation at *PGR* promoter B, 14 (93%) were classified as having high levels of PR, whereas 93 of 178 (52%) tumors that showed detectable DNA methylation at this promoter had high levels of PR protein (Table 2).

Analysis of DNA Methylation at *ESR1* Promoter C as a Continuous Measure and Expression of ERα and PR. In contrast to the other promoters studied, the wide range of DNA methylation PMR values for *ESR1* promoter C was amenable to analysis as a continuous variable. Using linear regression models, DNA methylation at *ESR1* promoter C was inversely associated with the degree of ERα (β = −0.26, P = <0.0001) and PR (β = −0.25, P = <0.0001) expression (Fig. 1). Controlling for age and site did not significantly alter the relationship between *ESR1* promoter C DNA methylation and protein expression (data not shown). In a model further adjusted by tumor characteristics (tumor size, grade, and nodal involvement) and tumor marker expression (ERα, PR, and HER-2/neu), elevated *ESR1* promoter C DNA methylation remained a significant predictor of expression of ERα (β = −0.18, P = 0.033). In this model, tumor size was the strongest predictor of promoter C methylation (β = −0.38, P = 0.002; other β estimates from the full model are not shown). The inverse relationship between ERα expression and DNA methylation of *ESR1* promoter C was similar among PR-positive tumors (β = −0.23, P = 0.035) and PR-negative tumors (β = −0.17, P = 0.062; P for interaction = 0.45; figures not shown). PR expression, however, was only associated with *ESR1* promoter C DNA methylation among ERα-positive tumors (β = −0.18, P = 0.034) but not among ERα-negative tumors (β = −0.05, P = 0.68), although the interaction term was not statistically significant (P = 0.40; figures not shown).

DNA Methylation, Tumor Characteristics, and Epidemiologic Risk Factors. DNA methylation at *ESR1* or *PGR* promoters was not associated with tumor size or nodal status (data not shown). Risk factors for breast cancer were not related to DNA methylation data when analyzed categorically (data not shown). When DNA methylation results for *ESR1* promoter C were analyzed as a continuous measure, age at first full-term birth (per 5 years) was weakly associated with degree of DNA methylation [multivariable β = −0.08; 95% confidence interval (95% CI), −0.15 to −0.01; Table 3]. After adjustment for levels of ERα expression, the β coefficient for age at first birth remained similar but the result was no longer statistically significant (β = −0.07; 95% CI, −0.14 to 0.003; other β estimates not shown). Other risk factors under study were not related to DNA methylation at *ESR1* promoter C (Table 3).

Discussion

Our analyses, based on unselected breast cancers ascertained in a population-based study, show that DNA methylation at CpG islands in *ESR1* (promoters A and B) and *PGR* (promoters A and B) is common in invasive breast cancer, but generally weak. DNA methylation at *PGR* promoters was most strongly related to decreased protein expression of ERα and PR. We also found that DNA methylation levels varied widely at *ESR1* promoter C, which contains a CpG shore (27) and has not been well studied.

ERα transcription is controlled by seven promoters in *ESR1* (35), of which two, promoters A and B, contain CpG islands. In cell lines, hypermethylation of these promoters is associated with lack of ERα expression (9, 10, 12), whereas treatment with a demethylation agent, 5-aza-2′-deoxycytidine, results in reexpression of ERα (13). However, methylation patterns in cell lines and human breast tumors may differ (11).

Previous analyses that used methylation-specific restriction enzymes in combination with Southern blotting or PCR (MSP) have suggested that methylation of promoter A is more frequent in ERα-negative than in ERα-positive tumors (7, 14); however, results have varied. Using MSP with six different primer sets, Lapidus and colleagues (18) detected DNA methylation in all 32 breast cancer tumors tested, irrespective of ER and PR status. However, a post hoc analysis based on subjective scoring of gels found "substantial methylation" in all 13 ERα-negative/PR-negative tumors, 8 of 11 (72.7%) ERα-positive/PR-negative tumors, and 4 of 11 (36.4%) ERα-negative/PR-negative tumors. Parrella and colleagues (19) were unable to confirm the aforementioned results using MSP and three of the same primer sets; they found DNA methylation at *ESR1* in 13 of 27 (48.1%) ERα-positive, 4 of 5 (80.0%) ER weakly positive, and 12 of 16 (75.0%) ERα-negative tumors. In an MSP analysis of frozen tumors, Li et al. found DNA methylation at *ESR1* in 49 of 54 (90.7%) ERα-negative tumors as compared with 108 of 134 (80.6%) ERα-positive tumors ($P = 0.04$); relationships were similar for PR expression. However, DNA methylation at *ESR1* was not significantly related to ERα protein levels when assessed biochemically. Finally, Hori et al. (17), in an analysis of 124 breast tumors using competitive PCR, found that methylation at two *ESR1* sites was unrelated to ER or PR status; however, methylation was associated with ERα-positive/PR-negative tumors.

Analyses using Southern blotting have also shown that DNA methylation occurs more frequently among ERα-negative as compared with ERα-positive tumors, although the method is less sensitive and the results have been inconsistent (13, 15, 16). Yoshida et al. (12) show figures suggesting that DNA methylation was detectable in some ERα-positive tumors. Fabianowska-Majewska et al. (16) reported that 45 of 62 (72%) tumors with detectable DNA methylation were ERα negative. Similarly, Iwase et al. (15) detected DNA methylation in 24 of 27 (89%) ERα-negative cancers as compared with 17 of 29 (59%) ERα-positive tumors. Finally, Lapidus et al. (13) found methylation in only 9 of 39 (23%) ERα-negative tumors and none of 53 ERα-positive tumors analyzed.

In summary, studies relating DNA methylation at *ESR1* promoter A to ERα status have been inconsistent, possibly reflecting differences in patient or tumor characteristics or the assays used for assessing DNA methylation or

Table 2. Relationships between DNA methylation PMR values and AQUA PR protein expression (200 breast cancer cases from the Polish Breast Cancer Study, 2000-2003)

Promoters	Low PR expression ($n = 86$)	High PR expression ($n = 107$)	P*
	n (%)	*n* (%)	
ESR1 promoter A			
0	14 (16.3)	13 (12.1)	
>0-5	72 (83.7)	94 (87.9)	
>5-50	0 (0.0)	0 (0.0)	
>50	0 (0.0)	0 (0.0)	0.53
ESR1 promoter B			
0	0 (0.0)	4 (3.7)	
>0-5	65 (75.6)	86 (80.4)	
>5-50	21 (24.4)	17 (15.9)	
>50	0 (0.0)	0 (0.0)	0.094
ESR1 promoter C			
0	0 (0.0)	0 (0.0)	
>0-5	3 (3.5)	6 (5.6)	
>5-50	65 (75.6)	91 (85.0)	
>50	18 (20.9)	10 (9.3)	0.066
PGR promoter A			
0	73 (84.9)	105 (98.1)	
>0-5	11 (12.8)	1 (0.9)	
>5-50	2 (2.3)	1 (0.9)	
>50	0 (0.0)	0 (0.0)	0.00057
PGR promoter B			
0	1 (1.2)	14 (13.1)	
>0-5	82 (95.3)	92 (86.0)	
>5-50	3 (3.5)	1 (0.9)	
>50	0 (0.0)	0 (0.0)	0.0013

NOTE: MethyLight results measure the level of DNA methylation at specific loci in test samples compared with a completely methylated DNA reference, yielding PMR for specific CpGs of target genes (32). PMR values were categorized as previously defined (32): no methylation (0 PMR), mild (>0-5 PMR), moderate (5-50 PMR), and heavy methylation (>50 PMR). TMAs of breast tumors were analyzed for ERα, PR, and HER-2/neu using AQUA, an automated quantitative immunofluorescent assay (22, 23), as previously described and categorized in a previous Polish Breast Cancer Study publication (24).
*Fisher's exact test.

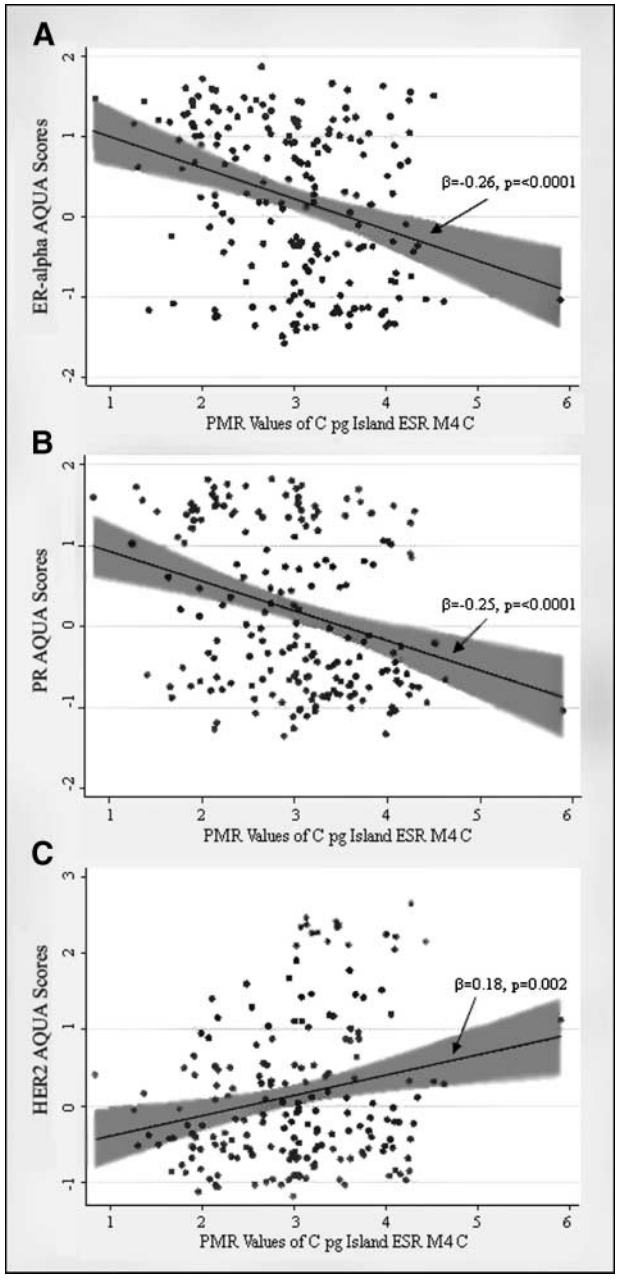

Figure 1. Scatterplot of PMR values of the CpG island *ESR1* M4 C and AQUA scores of ERα (**A**), PR (**B**), and HER2-neu (**C**) overlaid with the fitted linear regression line (β coefficient and corresponding *P* value provided) and 95% CI (shaded in gray).

ERα status. For example, studies based on frozen tissues are often skewed toward larger tumors. In addition, most methylation assays are nonquantitative. (36), although our data suggest that the range of methylation levels is narrow (30). Overall, our data, like the literature, overall suggest that *ESR1* promoter A is frequently (~85%) weakly methylated in breast cancer regardless of ER status (13, 15, 16, 18, 19, 37). These findings are consistent with the MethyLight study of Widschwendter et al. (33) that found methylation of *ESR1* promoter A (median PMR, 0.8) and showed a marginally significant association with ER status in 148 breast tumors ($P = 0.03$).

PGR (chr.11q22-23) encodes two isoforms, PR-A and PR-B, which are transcribed from two alternative promoters, A and B, with different functions; in cell lines, PR-B is required for the proliferative effect of progesterone and estrogen, whereas inactivation of PR-A contributes to repression of PR-B–mediated transcription (38-40). In our study, *PGR* promoter A was methylated in 7.7% and *PGR* promoter B in 92.2% of breast cancers, and DNA methylation at each promoter was significantly associated with reduced expression of total PR. Similarly, a study of 57 breast cancers using three methylation-specific enzymes with Southern blot analysis found that DNA methylation at any of the sites was associated with PR-negative status (13). Another report found that DNA methylation of *PGR* promoter B was related to PR-negative status by immunohistochemistry ($P = 0.03$; ref. 20). However, unlike our analysis, a previous study using MethyLight did not find that methylation of *PGR* promoter B was associated with PR status ($P = 0.18$), although overall levels of methylation were similarly weak (median PMR, 0.6; ref. 33). The reasons for the inconsistency are unknown.

In vitro data suggest that hypermethylation at *PGR* promoter regions may be a marker of sustained suppression of ERα expression (41). In the study of Leu et al. (41), repressed ERα expression in MCF-7 cells was associated with DNA methylation of *PGR* and reduced PR-A and PR-B transcripts. However, *PGR* hypermethylation occurred 36 hours after repression of ERα, suggesting that it was a late event that followed clonal expansion. Reactivation of PR expression required both ERα reexpression and *PGR* demethylation. Our data support these observations. We found that methylation of the promoter regions of PR-A and PR-B was more common among tumors with low expression of ERα (17.6% versus 1.7% and 97% versus 89.1%, respectively). Similarly, Widschwendter and colleagues (33) found that DNA methylation at *PGR* promoter B was the strongest predictor among 35 markers of ER status ($P = 0.0010$). Limited data suggest that *ESR1* promoter B methylation is more common in ERα-negative compared with ERα-positive tumors (12, 15), which is weakly supported in our study.

In addition, in evaluating CpG islands in *ESR1*, we also evaluated methylation at a CpG shore in promoter C. Levels varied widely at this locus and were statistically significantly associated with lower expression of ERα and PR. Confounding by age is an unlikely explanation for this result, given that methylation generally increases with age, and we observed a relationship with ERα-negative tumors, a disease more common among younger women. Few studies have examined the function of ERα promoter C (formerly referred to as promoter or the distal promoter) in controlling transcription. In small studies, up-regulation of transcription via promoter C was associated with increased levels of ERα protein in cell lines and human tumors (42) and methylation was related to down-regulation of transcription (12). Although one study has shown that promoter C related transcripts are detectable in normal breast tissue (42), these data were not evaluated in relation to methylation status. Although the exact role of hypermethylation of CpG shores is not confirmed, they have recently been shown to be highly conserved across species and to play a role in transcription of alternative

Table 3. Crude and multivariable-adjusted linear regression between known breast cancer risk factors and PMR DNA methylation values for *ESR1* promoter C (200 breast cancer cases from the Polish Breast Cancer Study, 2000-2003)

Risk factor	Crude model*		Multivariable-adjusted model†	
	β (95% CI)	P	β (95% CI)	P
No. of full-term births	0.03 (−0.07 to 0.13)	0.51	0.09 (−0.02 to 0.20)	0.12
Age at first birth (per 5-y increase)	−0.06 (−0.12 to 0.01)	0.14	−0.08 (−0.15 to −0.01)	0.030
Age at menarche	−0.04 (−0.11 to 0.03)	0.31	−0.02 (−0.10 to 0.05)	0.51
Age at menopause (per 5-y increase)	0.01 (−0.02 to 0.04)	0.45	0.02 (−0.13 to 0.16)	0.80
Menopause	0.16 (−0.19 to 0.50)	0.38	0.52 (−1.55 to 2.59)	0.62
Current BMI among premenopausal, per 5-unit increase	−0.03 (−0.29 to 0.23)	0.79	0.10 (−0.18 to 0.38)	0.47
Current BMI among postmenopausal, per 5-unit increase	−0.13 (−0.27 to 0.02)	0.083	−0.12 (−0.27 to 0.03)	0.12
Family history of cancer	0.04 (−0.36 to 0.45)	0.83	0.03 (−0.41 to 0.46)	0.91
History of benign breast disease	−0.06 (−0.35 to 0.22)	0.67	−0.12 (−0.42 to 0.17)	0.41

NOTE: MethyLight results measure the level of DNA methylation at specific loci in test samples compared with a completely methylated DNA reference, yielding PMR for specific CpGs of target genes (32). PMR values were categorized as previously defined (32): no methylation (0 PMR), mild (>0-5 PMR), moderate (5-50 PMR), and heavy methylation (>50 PMR).
*Crude models were adjusted for age (5-y categories) and site (Warsaw, Lodz).
†The multivariable-adjusted model included all the variables in the table as well as age, site, and education (less than high school graduate, high school graduate, and some college, professional training, or college graduate).

start sites (27). However, our results for this locus should be interpreted cautiously until additional data are available on the relationship between methylation status and protein expression in normal and diseased breast tissues.

Environmental factors are hypothesized to alter DNA methylation of specific genes (43-45), thus modifying breast cancer risk (44). This view is supported by the observation that as monozygotic twins age or spend more time apart, greater epigenetic differences emerge (46). We observed an association between older age at first birth and lower levels of hypermethylation of *ESR1* promoter C, which persisted after controlling for ERα expression. This result is consistent with data showing that acute estrogen exposure in animal models (47) and in humans (48) triggers DNA methylation of hormone-responsive genes, and with studies suggesting that delayed childbearing is more strongly linked to ERα-positive than to ERα-negative tumors. This study requires confirmation, but supports a possible role of environmental influences on epigenetic modifications (43).

In our study, hypermethylation of *ESR1* and *PGR* was unrelated to tumor size and nodal involvement, consistent with previous findings (15, 19, 37). Although ERα and PR expressions are positively related to each other, our methylation analysis did not shed conclusive light on these findings (49).

Strengths of our study include use of tumors from a population-based study, extraction of tumor-rich DNA, validation of our tissue sampling methods, and demonstration of assay reproducibility. Whereas we found consistent DNA methylation patterns within tumors, it is possible that random sampling error may explain some of our null results.

In conclusion, we found that methylation of *ESR1* and *PGR* was common, generally weak, and unlikely to account solely for the majority of ERα- and PR-negative breast tumors. We also found a preliminary suggestion that older age at first birth was more strongly associated with lower levels of hypermethylation of *ESR1* promoter C than with ERα expression, suggesting that DNA hypermethylation may be used to define some of the etiologic heterogeneity in breast cancer. However, to increase our knowledge of methylation in breast cancers, further studies in well-characterized, population-based cases are needed with consideration given to assaying of cell subpopulations within individual tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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