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CpG Island Tumor Suppressor Promoter Methylation in Non-BRCA-Associated Early Mammary Carcinogenesis

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Abstract

Background: Only 5% of all breast cancers are the result of *BRCA1/2* mutations. Methylation silencing of tumor suppressor genes is well described in sporadic breast cancer; however, its role in familial breast cancer is not known.

Methods: CpG island promoter methylation was tested in the initial random periareolar fine-needle aspiration sample from 109 asymptomatic women at high risk for breast cancer. Promoter methylation targets included *RARB* (M3 and M4), *ESR1*, *INK4a/ARF*, *BRCA1*, *PRA*, *PRB*, *RASSF1A*, *HIN-1*, and *CRBP1*.

Results: Although the overall frequency of CpG island promoter methylation events increased with age ($P < 0.0001$), no specific methylation event was associated with age. In contrast, CpG island methylation of RARB M4 ($P = 0.051$), *INK4a/ARF* ($P = 0.042$), *HIN-1* ($P = 0.044$), and *PRA* ($P = 0.032$), as well as the overall frequency of methylation events ($P = 0.004$), was associated with abnormal Masood cytology. The association between promoter methylation and familial breast cancer was tested in 40 unaffected premenopausal women in our cohort who underwent *BRCA1/2* mutation testing. Women with *BRCA1/2* mutations had a low frequency of CpG island promoter methylation (15 of 15 women had ≤4 methylation events), whereas women without a mutation showed a high frequency of promoter methylation events (24 of 25 women had 5-8 methylation events; $P < 0.0001$). Of women with a *BRCA1/2* mutation, none showed methylation of *HIN-1* and only 1 of 15 women showed CpG island methylation of *RARB* M4, *INK4a/ARF*, or PRB promoters.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Conclusions: This is the first evidence of CpG island methylation of tumor suppressor gene promoters in non-BRCA1/2 familial breast cancer.

Introduction

Transcriptional silencing of tumor suppressor genes (TSG) through methylation of CpG islands in promoter regions is thought to be an important early mechanism of human carcinogenesis (1,2). Growing evidence suggests that epigenetic inactivation via cytosine methylation plays a role in the transformation of normal cells to cancerous cells, underscoring the need to investigate global CpG island methylation patterns (1). Building on these observations, Toyota et al. proposed that cancer may develop through the simultaneous inactivation of multiple TSGs and induction of mismatch repair deficiency (3). In studies of colorectal cancer, an established panel of specific promoters [methylated in tumors-1 (*MINT1*), methylated in tumors-2 (*MINT2*), methylated in tumors-31 (*MINT31*), *INK4a/ARF*, and *hMLH1*] has been used to distinguish between low-frequency methylation (0 or 1 of 5 markers methylated) and high-frequency methylation (≥2 of 5 markers methylated; refs. 4-7). Studies by Weisenberger et al. tested 200 methylation markers in 295 colon cancer specimens (8).

Two types of methylation patterns have been reported in colorectal cancer: type A for aging-specific methylation and type C for cancer-specific methylation (9). Type A methylation is characterized by a high incidence of CpG island methylation in tumors accompanied by a slight incidence in the detection of methylation in normal colon mucosa as well (3). Type C methylation, by contrast, occurs exclusively in a subset of colorectal cancers and at a lower frequency than type A methylation (3). Type A methylation is thought to increase susceptibility of aging cells to become predisposed to transformation, whereas type C methylation may contribute to neoplastic progression in a subset of cases (9). Type C methylation in colorectal cancer is observed for *INK4a/ARF*, thrombospondin-1 (*THBS1*), a *p53*-inducible angiogenesis inhibitor, and the mismatch repair gene *hMLH1* (3,9).

Translational Relevance

While identification of BRCA1/2 mutations represents a major milestone in understanding inherited breast cancer, only 5% of all breast cancers are the result of such mutations. Methylation silencing of tumor suppressor genes is well described in sporadic breast cancer; however, the role for methylation silencing of tumor suppressor genes in familial breast cancer is not known. Here, we provide the first evidence of CpG island methylation of tumor suppressor gene promoters during breast cancer initiation, as well as in non-BRCA1/2 familial breast cancer. We evaluated the methylation status of 10 promoter CpG islands in mammary cytology from high-risk women. We observed a bimodal distribution of promoter methylation events in mammary cytology from this cohort. Promoter methylation of the retinoic acid receptor-β (RARB) M4, INK4a/ARF, HIN-1, and progesterone receptor α (PRA) predicted mammary atypia in high-risk women. Whereas the total number of promoter methylation events was associated with increasing age, there was no association between any single CpG island methylation event and increasing age. We also tested for the association between individual promoter methylation events and the presence or absence of a BRCA1/2 mutation in unaffected women with a high pretest probability of carrying a mutation. There was a significant association between the number of CpG island methylation events and absence of a BRCA1 or BRCA2 mutation. These data provide the first evidence for CpG island promoter methylation of tumor suppressor genes in non-BRCA-associated familial mammary carcinogenesis.

Whereas the existence of type C tumor suppressor promoter methylation in colorectal cancer is well described, the existence of nonrandom tumor suppressor promoter methylation events

in breast cancer is unclear (10-12). Huang et al. performed a genome-wide screening of 276 CpG island loci in a group of breast cancer cell lines using differential methylation hybridization, a novel array-based method, and found that preexisting methylation within CpG island loci may stimulate subsequent *de novo* methylation in cancer cells (11). Thus, they hypothesize that certain loci are more susceptible than others to becoming methylated in breast cancer cells. Bae et al. investigated the methylation profile of 12 genes in 109 invasive breast tumors (representing ductal, lobular, and mucinous histologic subtypes) and concluded that methylation frequency for all three histologic subtypes does not support the existence of type C tumor suppressor promoter methylation in breast cancer (10). Several other studies have investigated various panels of methylation markers in breast cancer and provide evidence of significant association among various methylated loci, suggesting a nonrandom distribution of promoter methylation in mammary carcinogenesis (12,13). Notably, Parrella et al. found that if estrogen receptor-a (*ESR1*) promoter was methylated, then E-cadherin (*CDH1*), glutathione *S*-transferase (*GSTP1*), cyclin D2 (*CCND2*), and thyroid hormone receptor-β1 (*TRB1*) promoters were also likely to be methylated independent of the overall methylation frequency (13).

Here, we tested for promoter methylation in early mammary carcinogenesis by analyzing a panel of 10 CpG islands of candidate TSGs in mammary epithelial cells from 109 asymptomatic women at increased risk for breast cancer. We chose our set of methylation markers based on relevance of the gene to mammary carcinogenesis, lack of methylation in stroma, and presence of promoter methylation in early mammary carcinogenesis [e.g., atypical hyperplasia, ductal carcinoma *in situ* (DCIS), and lobular carcinoma *in situ* (LCIS)]. Based on these criteria, we chose genes that were critical for (*a*) hormone signaling such as *ESR1* (14-16), progesterone receptor (*PR*; refs. 15,17), retinoic acid receptor-β (*RARB*; refs. 18-23), and cellular retinol-binding protein 1 (*CRBP1*; refs. 24,25), (*b*) cellular proliferation such as the cytokine high in normal-1 (*HIN-1*; refs. 19,20,26), cell cycling regulators such as cyclin-dependent kinase inhibitor 2A (*INK4a/ARF*; refs. 27-31), and cell signaling intermediates such as Ras-association domain family protein 1 isoform A (*RASSF1A*; refs. 19,20,32-34), and (*c*) DNA repair genes such as breast cancer associated-1 gene (*BRCA1*; refs. 35-40).

The frequency of promoter methylation was tested using samples derived from random periareolar fine-needle aspiration (RPFNA). RPFNA is a research technique developed to repeatedly sample mammary cells from the whole breast of asymptomatic women at high risk for development of breast cancer to assess both breast cancer risk and response to chemoprevention (18,41,42). RPFNA can be done successfully in a majority of high-risk women (82-89% cell yield; refs. 18,41,42). RPFNA samples were classified using the Masood cytology index to indicate the level of cytologic abnormality. We also tested these samples for the association between CpG island promoter methylation and the presence or absence of a *BRCA1/2* mutation in women with a high pretest probability of carrying a mutation.

Materials and Methods

Informed Consent

The study was approved by the Human Subjects Committee and Institutional Review Board at Duke University Medical Center in accordance with assurances filed with and approved by the Department of Health and Human Services.

Subject Recruitment

Subjects were (*a*) recruited on entry to the Duke University High-Risk Clinic or (*b*) women who were undergoing surgery for stage I or II breast cancer. Entry to the Duke High-Risk Clinic is defined as individuals with one of the following: (*a*) a 5-year Gail model risk score ≥1.7%,

(*b*) a prior biopsy exhibiting atypia, LCIS, or DCIS, and (*c*) known or suspected *BRCA1/2* mutation carrier (42). Women undergoing surgery for stage I or II breast cancer underwent aspiration of the opposite breast only in the operating room; no woman had chemotherapy before aspiration. Women in the Duke High-Risk Clinic were initially approached by Dr. V.L. Seewaldt or her physician assistant and then consented by a study nurse or coordinator; 154 women were approached and 94 (61%) agreed to participate. Women who underwent aspiration in the operating room were initially approached by surgeon Dr. L.G. Wilke and consented by either Dr. L.G. Wilke or our study nurse or coordinator.

Eligibility

To be eligible for RPFNA, high-risk women were required to have at least one of the following major risk factors for breast cancer: (*a*) 5-year Gail risk calculation >1.7%, (*b*) prior biopsy exhibiting atypical hyperplasia, LCIS, or DCIS, or (*c*) known or suspected *BRCA1/2* mutation carrier (42). Women undergoing RPFNA in the operating room were required to have either stage I or II breast cancer and required to not have neoadjuvant chemotherapy. To be eligible for the high-risk BRCA1/2 analysis, subjects were required to be (*a*) unaffected and (*b*) have a 5% probability of having a BRCA1/2 mutation by either the BRCA1PRO or the BRCA2PRO model (see below).

Mathematical Assessment of Breast Cancer Risk

BRCAPRO score and Gail model assessments were done using the CancerGene software and Breast Cancer Risk Assessment Tool.[7] The 5-year breast cancer risk calculated by the Gail model identifies women who are at increased risk compared with their age- and race-matched peers (43). Women ages <35 years are not appropriate for Gail risk calculation. We did not perform Gail risk calculation for African American women because of the potential underestimation of risk in this population. The BRCAPRO model calculates the probability of an individual carrying a mutation in the BRCA1 or BRCA2 genes using Bayesian methods to incorporate relevant family history of breast and/or ovarian cancers, including second-degree relatives (44).

RPFNA

RPFNA was done as published previously (18,42,45), in accordance with methods established and validated by Fabian et al. (42). Each RPFNA sample consists of a pool of 10 needle aspirates from a single, unaffected breast, that is, one to two RPFNA samples were collected per woman. The presence of atypia in RPFNA cytology obtained from pooled aspirates is prospectively validated to predict a 5.6-fold increase in breast cancer risk in high-risk women (42). Single RPFNA needle aspirates are not usually tested, as these measurements are not validated to predict risk (42). A minimum of one epithelial cell cluster with at least 10 epithelial cells was required to sufficiently determine pathology; the most atypical cell cluster was examined and scored (41,42). Cells were classified qualitatively as nonproliferative, hyperplasia, or hyperplasia with atypia (46). Cytology preparations were also given a semiquantitative index score through evaluation by the Masood cytology index (47). As described previously, cells were given a score of 1 to 4 points for each of six morphologic characteristics that include cell arrangement, pleiomorphism, number of myoepithelial cells, anisonucleosis, nucleoli, and chromatin clumping; the sum of these points computed the Masood score: ≤10, nonproliferative (normal); 11-13, hyperplasia; 14-17, atypia; and >17, suspicious cytology (42,47). The number of epithelial cells was quantified and classified as <10 (insufficient quantity for cytologic analysis), 10-100, 100-500, 500-1,000, 1,000-5,000,

[7]CancerGene software and Breast Cancer Risk Assessment Tool are available online at http://www4.utsouthwestern.edu/breasthealth/cagene/ and http://www.cancer.gov/bcrisktool/, respectively.

and >5,000 cells. Morphologic assessment, Masood cytology index scores, and cell count were assigned by a blinded, single dedicated pathologist (C.Z.; ref. 42).

Masood and Methylation Assessment of Individuals Undergoing Unilateral or Bilateral RPFNA

Twenty-five of 109 women who had either (*a*) prior mastectomy and/or radiation therapy for DCIS (9 women) or (*b*) concurrent surgery for breast cancer (16 women) underwent unilateral RPFNA (contralateral breast only). Eighty-four of 109 women who did not have (*a*) mastectomy, (*b*) radiation therapy, or (*c*) contralateral surgery for breast cancer underwent bilateral RPFNA. For women with bilateral RPFNA, the sample with the highest Masood and cell count was considered for this analysis. Masood score and promoter methylation were derived from the same RPFNA sample.

Materials and Cell Culture Lines

Sodium bisulfite (Sigma; A.C.S.) and hydroquinone (Sigma; >99%) were used under reduced lighting and stored in a desiccator. 2-Pyrrolidinone was purchased from Fluka (>99%). Q-Solution is a proprietary reagent supplied as a 5× solution that comes with Qiagen HotStarTaq DNA polymerase. The AG11134 normal human mammary epithelial cell line was purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute). The HMEC1001-15 and HMEC1001-16 normal human mammary epithelial cell lines were purchased from Cambrex. All normal cell lines were grown in mammary epithelial cell basal medium as described previously (48). The MCF7-LXSN breast cancer cell line was established by V.L. Seewaldt and is described previously (49). The HMEC-SR cell line is described previously (18). Breast cancer cell lines were grown in supplemented α-MEM (Life Technologies; ref. 49).

DNA Extraction and Bisulfite Treatment

DNA was extracted from breast cancer cell lines and RPFNA as published previously; bisulfite treatment was as published previously (18).

Methylation-Specific PCR

All methylation-specific PCR (MSP) consisted of 50 ng bisulfite-treated DNA, 1× PCR buffer, 250 μmol/L of each deoxynucleotriphosphate, 200 nmol/L of each primer, and 2.5 units HotStarTaq polymerase (Qiagen) in 30 μL total volume. PCR buffers were individually optimized for the methylated and unmethylated programs using CpGenome Universal Methylated or Unmethylated Control DNA (Chemicon). A GeneAmp PCR System 9700 (Applied Biosystems) or iCycler (Bio-Rad) was used for all amplifications. MSP cycling conditions consisted of 95°C for 5 min followed by 40 amplification cycles (94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min) followed by a final extension of 4 min at 72°C. PCR products were visualized on 1.5% ethidium bromide agarose gels using an Image Station 440 (Carestream Health). To estimate PCR sensitivity, titrated experiments were done using known amounts of methylated genomic DNA (1 μg-100 pg) spiked in unmethylated genomic DNA for a total of 1 μg (Supplementary Data; refs. 14, 18, 27, 35). Ten MSP promoter methylation targets were tested: *RARB* at M3 (nucleotides −51 to +162), *RARB* at M4 (nucleotides +104 to +251; ref. 18), *BRCA1* (nucleotides −150 to +32; ref. 50), ESR1 (nucleotides +357 to +474; ref. 16), *INK4a/ARF* (nucleotides +171 to +312; refs. 27, 51), *PRA* (nucleotides +910 to +1008; refs. 52, 53), *PRB* (nucleotides +156 to +355; refs. 52, 53), *RASSF1A* (nucleotides −73 to +97; ref. 33), *HIN-1* (nucleotides −231 to −37; ref. 54), and *CRBP1* (nucleotides −45 to +65; ref. 55). Nucleotide positions are relative to the transcriptional start site for each gene. MSP primers and conditions are listed in Table 1.

Sequenom MassARRAY Quantitative Methylation Analysis

Sequenom MassARRAY platform was used to perform quantitative methylation analysis (56). This system uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in conjunction with RNA base-specific cleavage (MassCLEAVE) for the detection and quantitative analysis of DNA methylation (56). Genomic DNA (1 μg) was bisulfite treated as described previously (18). The primers were designed and prevalidated by Sequenom (Sequenom Standard EpiPanel). Each reverse primer has a T7 promoter tag for *in vitro* transcription (5′-cagtaatacgactcactatagggagaaggct-3′) and the forward primer is tagged with a 10-mer to balance melting temperature (5′-aggaagagag-3′). PCR consisted of 1 μL bisulfite-treated DNA, 1× PCR buffer, 250 μmol/L of each deoxynucleotriphosphate, 200 nmol/L of each primer, and 2.5 units HotStarTaq polymerase (Qiagen) in 5 μL reaction volume. Cycling conditions consisted of 94°C for 15 min followed by 45 amplification cycles (94°C for 20 s, annealing temperature for 30 s, and 72°C for 1 min) and by a final extension of 4 min at 72°C. After shrimp alkaline phosphatase treatment, 2 μL of the PCR product was used as template for *in vitro* transcription and RNase A cleavage for the T-reverse reaction as per manufacturer's instructions (Sequenom hMC). The samples were desalted and spotted on a 384-well SpectroCHIP (Sequenom) using a MassARRAY nano-dispenser (Samsung) followed by spectral acquisition on a MassARRAY Analyzer Compact matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom). The spectra's methylation ratios for each CpG site or an aggregate of multiple CpG sites were generated by the MassARRAY EpiTYPER software version 1.0 (Sequenom). Seven RPFNA samples as well as CpGenome Universal Methylated and Unmethylated Control DNA (Chemicon) were analyzed. The sequences of the primers used are available on request.

Statistical Methods

The Wilcoxon rank-sum test was used to compare the mean ranks of each covariate (median age, body mass index, Gail model score, probability of *BRCA1/2* mutation, and Masood score) according to positive or negative marker methylation status. Independently, the proportion of premenopausal and Caucasian women with a methylated marker was compared using the Pearson χ^2 test.

Hierarchical clustering of CpG island methylation events was done in the R statistical environment using complete linkage of correlations for symmetric binary data (10). Pairwise correlations in gene methylation were examined using Fisher's exact test. To correct for the 45 multiple comparisons, *P* values are adjusted using the Benjamini step-up method for controlling the false discovery rate (57).

The Wilcoxon rank-sum test was used to compare the mean ranks of the number of CpG island methylation events, age, and BRCAPRO scores in subjects testing negative as opposed to testing positive for *BRCA1/2* mutation. The correlation between the number of CpG island promoter methylation events and body mass index, age, Gail model score, and probability of an individual having a *BRCA1/2* mutation (BRCAPRO score) was tested using the Spearman rank correlation coefficients. The Wilcoxon rank-sum test was used to test for differences in the mean ranks of the number of positive CpG island markers in Caucasians compared with African Americans as well as premenopausal compared with perimenopausal/postmenopausal women.

Results

Study Demographics

We tested the initial RPFNA sample from 109 women who (*a*) underwent RPFNA at Duke University Medical Center from March 1, 2003 to October 1, 2007 and (*b*) had sufficient

epithelial cells for cytologic testing. Study subject demographics are listed in Table 2. Seventy-seven percent (84 of 109) of subjects had bilateral RPFNA. Unilateral RPFNA was done on women with prior mastectomy and RPFNA was not done on radiated breast tissue; therefore, 23% (25 of 109) of subjects had unilateral RPFNA. Ninety percent (98 of 109) of the women were Caucasian and 10% (11 of 109) were African American. Twenty-nine unaffected premenopausal women with a familial pattern of breast cancer underwent *BRCA1/2* mutation testing; 34% (10 of 29) of women tested positive for either *BRCA1* (8 of 10) or BRCA2 (2 of 10) mutation.

CpG Island Methylation Analysis of RPFNA Cytology

We tested for CpG island promoter methylation of 10 breast cancer-associated genes in the initial RPFNA cytology from 109 high-risk women; 193 RPFNA samples were tested. CpG island promoter targets included *RARB* (M3 and M4 sites), *ESR1*, *INK4a/ARF*, *BRCA1*, *PRA*, *PRB*, *RASSF1A*, *HIN-1*, and *CRBP1*. To perform this analysis, subjects were considered methylated for an individual marker if CpG island promoter methylation was detected in RPFNA cytology from either one breast (unilateral methylation) or both breasts (bilateral methylation). Representative RPFNA promoter methylation testing is presented in Fig. 1A. The distribution of each CpG island marker is presented in Table 3 and Fig. 1B. The median number of positive CpG island promoter methylation events per individual was 4 and the mean number was 3.75. A total of 19 methylation markers in 4 samples had missing methylation data (see Table 3). This was due to lack of amplification of the unmethylated control for the specific sample. We considered the sample “inadequate” for analysis. Among the 10 genes tested, the most frequently methylated CpG island markers in RPFNA cytology from high-risk women were *PRA* (125 of 190; 65.8%) and *RARB* M3 (112 of 193; 58.0%). The least frequently methylated CpG island markers were *HIN-1* (29 of 190; 15.3%) and *PRB* (30 of 190; 15.8%).

Bimodal Distribution of Promoter Methylation

The distribution of promoter methylation events is shown in Fig. 1C. We observe a bimodal distribution of methylation events in RPFNA cytology from high-risk women. Specifically, two peaks are observed in a plot of the sum of methylation events observed per individual versus the number of women having that number of methylation events. This is significant in that not all risk is expected to be associated with high frequency of CpG island promoter methylation.

Specific Promoter Methylation Events Are Associated with Abnormal Masood Cytology Index but Not Age

Two types of methylation patterns are described previously in colorectal cancer: aging-specific methylation and cancer-specific methylation (9). We tested for age-related methylation and whether specific promoter methylation events predicted abnormal RPFNA cytology. Promoter methylation in RPFNA cytology from 109 subjects was compared with age and Masood cytology index score (Table 4). Although overall methylation events increased with age ($P < 0.0001$), no specific methylation marker was associated with increasing age. In contrast, methylation of *RARB* M4 ($P = 0.051$), *INK4a/ARF* ($P = 0.042$), *HIN-1* ($P = 0.044$), and *PRA* ($P = 0.032$), as well as the overall number of methylation events ($P = 0.004$), was associated with increased Masood cytology index score. These data show that whereas the overall frequency of methylation events increases with age (Table 4), promoter methylation of *RARB* M4, *INK4a/ARF*, *HIN-1*, and *PRA* is associated with abnormal Masood cytology in mamma-ry epithelial cells from high-risk women (Table 4). The results of this exploratory analysis provide evidence that specific promoter methylation events are associated with early mammary carcinogenesis.

Hierarchical Cluster of RPFNA Promoter Methylation

The clustering of methylation patterns in the 10 genes was generated from all observations, including bilateral samples, which are without missing values (n = 189). A symmetric binary distance was used as the measure of pairwise correlation and complete linkage was used to build the agglomerative tree structure (Fig. 2A). We observed hierarchical clustering of *RARB* M4, *HIN-1*, *PRB*, and *INK4a/ARF*. As described above, promoter methylation of *RARB* M4, *HIN-1*, *PRB*, and *INK4a/ARF* was also associated with increased Masood cytology index (Table 4).

Associations between Methylation Markers

The association between all pairwise combinations of methylation markers was examined in the 189 samples through Fisher's exact test for the odds ratio. Fig. 2B provides the estimated odds ratio and adjusted P values for all 45 comparisons. After correcting for the false discovery rate at the $\alpha = 0.05$ level, specific associations were observed between promoter methylation of (*a*) *RARB* M3 and *BRCA1*, *PRB*, and *CRBP1* and (*b*) *RASSF1A* and *HIN-1*.

Associations between Promoter Methylation and Clinical Variables

We tested the association between promoter methylation and menopausal status, race, Gail model risk score, and BRCAPRO model score (Table 4). Due to the limitations of the Gail model, only 61% (67 of 109) of subjects could be assessed. We did not calculate a Gail model score in 42 individuals due to a prior history of contralateral breast cancer or LCIS/DCIS, the subject being age <35 years, or the Gail model underestimating risk in African American subjects. There was no statistically significant association found between the overall number of promoter methylation events and race (P = 0.68), menopausal status (P = 0.12), Gail model risk score ($r = -0.024$, P = 0.85), or BRCAPRO model score (BRCA1PRO: $r = -0.074$, P = 0.41; BRCA2PRO: r = 0.003, P = 0.97). There was a significant association between lack of *INK4a/ARF* promoter methylation and both BRCA1PRO and BRCA2PRO scores (P = 0.010 and 0.031, respectively).

CpG Island Promoter Methylation Is Observed in Unaffected Women Who Test Negative for a BRCA1/2 Mutation

We tested women with a familial pattern of breast cancer to observe whether there was an association between CpG island promoter methylation frequency and the presence or absence of a *BRCA1/2* mutation. Forty unaffected women in the cohort underwent *BRCA1/2* mutation testing. To be eligible for *BRCA1/2* mutation testing, women were required to have a pretest probability of ≥0.05 by either the BRCA1PRO or the BRCA2PRO model. The 40 high-risk women we tested (*a*) were premenopausal, (*b*) had significant family history of breast cancer, and (*c*) were unaffected. Twenty-five of 40 women tested negative for both *BRCA1* and *BRCA2* mutations; 15 of 40 women tested positive for either a *BRCA1* or a *BRCA2* mutation. The distribution of CpG island methylation events for women with or without *BRCA1/2* mutation is shown in Fig. 3, and the number and percentage of CpG island methylation events for 15 women testing positive for *BRCA1/2* mutations is shown in Table 5. In the group of women testing positive for *BRCA1/2*, 15 of 15 women had ≤4 of 10 CpG island promoter methylation events, whereas only 1 of 25 woman testing negative for *BRCA1/2* had ≤4 of 10 CpG island promoter methylation events ($P < 0.0001$). Of women with a *BRCA1/2* mutation, none showed methylation of *HIN-1* promoter and 1 of 15 women showed methylation of each of the following promoters: *RARB* M4, *INK4a/ARF*, or *PRB*. In contrast, 8 women with a *BRCA1/2* mutation exhibited methylation of *PRA* and 6 women with a *BRCA1/2* mutation exhibited methylation of *RARB* M3. The median age of women testing positive for *BRCA1/2* was 41 years (range, 29-45), whereas the median age for those testing negative was 44 years (range, 30-48; P = 0.43).

We also tested for CpG island promoter methylation in 10 low-risk individuals who underwent benign breast procedures. The median age was 41.6 years (range, 33-59), median 5-year Gail model risk score was 0.92 (range, 0.6-1.5), no woman had taken hormone therapy, and no woman had a prior breast biopsy with atypia, DCIS/LCIS, or cancer. The median number of methylation events was 2.6/10 (range, 0-5); 9 of 10 women had ≤3 promoter methylation events and 1 of 10 had 5 promoter methylation events. The distribution of methylation events for these low-risk women was 8 of 10 *PRA*, 7 of 10 *RARB* M3, 3 of 10 *INK4a/ARF*, 2 of 10 *CRBP1*, 2 of 10 *RASSF1A*, 2 of 10 *PRB*, and 0 of 10 for *ESR1*, *BRCA1*, and *HIN-1*. These observations provide preliminary evidence that, in unaffected high-risk women with a familial pattern of inherited breast cancer, there is an inverse association between frequency of promoter methylation events and presence of a *BRCA1/2* mutation.

Comparison between Sequenom Analysis and Conventional MSP

Sequenom analysis of promoter methylation was compared with conventional MSP for a limited number of RPFNA cytology specimens (Fig. 4). Sequenom preferred primers (Sequenom Standard EpiPanel) have not been developed for *PRA*, *PRB*, *HIN-1*, and *CRBP1* so analysis was done only for *RARB* M3 and M4, *ESR1*, *BRCA1*, *RASSF1A*, and I*NK4a/ARF*. MSP was optimized for all 10 methylation markers to detect 1 copy of the methylated marker gene among 10,000 copies of unmethylated DNA (0.01%; Supplementary Data). The sensitivity of Sequenom is 5 copies of methylated DNA among 100 unmethylated DNA copies (5.0%). There was a significant difference in the number of methylation events detected by conventional MSP versus Sequenom analysis for *RASSF1A* (7 of 7 positive versus 1 of 7 samples with 1 CpG site ≥20% methylated) and *INK4a/ARF* (5 of 7 positive versus 0 of 7 samples with >20% methylation). In contrast, there was better concordance for conventional MSP versus Sequenom analysis for *ESR1* (4 of 7 positive versus 2 of 7 samples with ≥1 CpG site ≥20% methylated) and *RARB* M3 (7 of 7 positive versus 3 of 7 samples with ≥1 CpG site ≥20% methylated). These differences likely reflect the heterogeneous nature of RPFNA cytology and the need to optimize primer conditions with a high degree of sensitivity.

Discussion

Tumorigenesis is hypothesized to be a multistep process resulting from the accumulation of genetic losses and epigenetic changes. A multitude of studies using the candidate gene approach have established the importance of DNA promoter methylation in TSG silencing during early mammary carcinogenesis; however, the contribution of CpG island promoter methylation in non-BRCA-mediated carcinogenesis is unclear.

Our exploratory analysis of women at high-risk for breast cancer provides preliminary evidence for CpG island promoter methylation in non-BRCA-mediated carcinogenesis. We found that although the overall frequency of methylation events increased with age ($P < 0.0001$), there was no association between age and any individual promoter methylation event. Importantly, we also observe (*a*) a bimodal distribution of promoter methylation events in mammary epithelial cells from high-risk women, which is expected because not all risk is hypothesized to result from a high frequency of promoter methylation events, and (*b*) an association between abnormal Masood cytology and methylation of *RARB* M4 ($P = 0.051$), *INK4a/ARF* ($P = 0.042$), *HIN-1* ($P = 0.044$), and *PRA* ($P = 0.032$) promoters. Because the presence of atypia in RPFNA is associated with a 5.6-fold independent short-term breast cancer risk, these data provide evidence that specific CpG island promoter methylation events are associated with early mammary carcinogenesis.

In this study, we observe promoter methylation in RPFNA cytology of phenotypically normal cells, including nonproliferative (normal; Masood score ≤ 10) and hyperplastic (Masood score 11-13) mammary epithelial cells. However, our cohort consists of women who are at high risk

for breast cancer and a significant number of these women have a prior biopsy showing premalignant and malignant mammary changes. Recent studies show loss of expression of key TSGs such as *RARB* and *INK4a/ARF* (18,27). Taken together, these observations provide evidence that CpG island promoter methylation in phenotypically normal cells from high-risk women may be an expected finding, particularly in women with a history of premalignant or malignant breast biopsy.

In premenopausal women with a familial pattern of breast cancer, we observe an association between total number of promoter methylation events and the presence or absence of a *BRCA1/2* mutation. The 40 high-risk women we tested for *BRCA1/2* mutations (*a*) were premenopausal, (*b*) had a strong family history of breast cancer, and (*c*) were unaffected. Of the 15 women testing positive for *BRCA1/2* mutations, 100% had ≤4 methylated promoter events, whereas only 1 of the 25 women testing negative for *BRCA1/2* mutations had ≤4 CpG island promoter methylation events ($P < 0.0001$). Consistent with prior observations by Krop et al., no woman with a *BRCA1/2* mutation showed methylation of *HIN-1* (54). We observed previously that the frequency of *INK4a/ARF* promoter methylation was inversely associated with the likelihood of an individual carrying a *BRCA1* or *BRCA2* mutation as measured by BRCAPRO (27). Consistent with this observation, only one woman with a *BRCA1/2* mutation showed CpG island promoter methylation of *INK4a/ARF*, *RARB* M4, or *PRB*. These observations provide evidence that unaffected women with a *BRCA1/2* mutation have an overall low frequency of CpG island promoter methylation events (≤4 of the 10 markers tested) relative to high-risk unaffected women who test negative for a *BRCA1/2* mutation and that they do not frequently exhibit methylation of *HIN-1*, *INK4a/ARF*, *RARB* M4, or *PRB* promoters.

In our samples, we observed hierarchical clustering of *RARB* M4, *INK4a/ARF*, *PRB*, and *HIN-1* promoter methylation events. Dysregulation and loss of expression of *RARB*, p16 (*INK4a/ARF*), and *HIN-1* are known to play key roles in mammary carcinogenesis. *RARB* is an important regulator of proliferation and apoptosis in mammary epithelial cells and a tumor suppressor in breast cancer. The *RARB* promoter has been shown to be hypermethylated in early mammary carcinogenesis and predicts an aggressive phenotype in salivary gland cancer (18,21,23,49,58-60). Likewise, p16 (*INK4a/ARF*) has been established as a key regulator of cell cycle progression and senescence (61,62). Cultured human mammary epithelial cells that lack p16 (*INK4a/ARF*) activity have been shown to exhibit premalignant phenotypes such as telomeric dysfunction, centrosomal dysfunction, a sustained stress response, and, most recently, a dysregulation of chromatin remodeling and DNA methylation (63,64). The progesterone receptor is a steroid hormone receptor expressed as two isoforms, α (*PRA*) and β (*PRB*), which mediates the effects of progesterone (17,65). Both isoforms are coexpressed in hormonally receptive tissues such as breast, endometrium, and ovary and have distinct transcriptional activities (17,65). Mote et al. found that, in normal breast, expression levels of *PRA* and *PRB* are comparable, but a high proportion of atypical lesions predominantly expressed only one of the two isoforms (usually *PRA* predominated) and that this phenomenon is an early event in breast carcinogenesis (17). Exclusive expression of *PRB* was only observed rarely (11%) in *BRCA1* carriers and was never seen in *BRCA2* carriers. In summation, they suggest that this altered balance of progesterone receptor isoforms leads to altered regulation of differentiation in cells that consequently progress into invasive lesions (66). *HIN-1* is a putative cytokine and candidate TSG (26). Its expression is markedly decreased in a majority of primary breast tumors and preinvasive lesions. Studies have shown that the silencing of *HIN-1* is most likely due to epigenetic mechanisms rather than genetic alterations of the gene (26,67). High expression of *HIN-1* in organs composed of branching ductal epithelia suggests that it may play a role in regulating epithelial cell proliferation, differentiation, or morphogenesis (26).

CRBP1 is a retinol transport protein down-regulated in breast cancer cells (24). Bistulfi et al. showed that when retinoic acid signaling was impaired in HME1 cells, *CRBP1* became transcriptionally inactive and suggested a repressive “domino effect” whereby *RARB* first becomes transcriptionally silenced followed by *CRBP1* (24). Our study's findings support the theory of this sequential epigenetic silencing phenomenon. Thirty-six of 40 (90%) subjects with CpG island promoter methylation of *CRBP1* also displayed methylation of the *RARB* promoter (M3 and/or M4 site). However, of 81 subjects with *RARB* methylation, only 36 (44%) displayed *CRBP1* methylation. Thus, our study supports the assertion by Bistulfi et al. that *RARB* promoter methylation precedes *CRBP1* promoter methylation.

Our studies using RPFNA from high-risk women contrast with studies done by Bae et al. in breast cancer biopsy specimens obtained from both Korean and American women (10). Notably, our studies were done in high-risk American women and included a significant percentage of women with a familial pattern of breast cancer inheritance. There are also important differences in the panel of methylation markers tested in each study. We tested for CpG island promoter methylation of *RARB* (M3 and M4), *INK4a/ARF*, and *HIN-1*. Although Bae et al. tested for CpG island promoter methylation of *RARB* M3 and *HIN-1*, their studies did not test for *RARB* M4 and *INK4a/ARF* promoter methylation. Although both studies failed to find an association between breast cancer initiation and promoter methylation of *RARB* M3, *BRCA1*, *ESR1*, or *RASSF1A*, they report a relatively unimodal distribution of methylation frequency for ductal, lobular, and mucinous cancer. In contrast, our study shows a bimodal distribution of methylation frequency in women at high risk for developing breast cancer.

The lack of concordance between our conventional MSP and Sequenom results of a selected group of RPFNA samples are likely due to several limitations. First, the preferred Sequenom primers for *RARB*, *ESR1*, *BRCA1*, *RASSF1A* and *INK4a/ARF* did not span the same number of CpG sites as those spanned by our conventional MSP primers. Second, when adjacent CpG sites fall within one fragment or when fragment masses are overlapping, the resulting methylation ratios are actually and average of the methylation levels for the aggregate sites. No useful quantitative methylation information can be obtained from these CpG sites. In addition, cleavage products that fall outside of the spectral range of the mass spectrometer (1,000-11,000 Da) escape detection, thus underestimating the methylation levels of gene promoters, such as those reported for breast cancer and adjacent normal breast tissues (68). A third, key limitation of Sequenom is low sensitivity. Sensitivity of Sequenom is 5% versus 0.01% for MSP optimized in our laboratory. This issue becomes particularly important during analysis of small heterogeneous samples such as RPFNA. Pyrosequencing is another available method of quantitative methylation analysis that may help to overcome the limitations of Sequenom technology mentioned above. In one recent study, methylation levels obtained by Pyrosequencing and MSP showed high correlation when assessing the hyper methylation of *RARB* and *RASSF1A* promoters in salivary gland carcinomas (58). However, the sensitivity of Pyrosequencing is still significantly less than that achieved by our conventional MSP analysis. Because breast cancer is likely to arise from the most abnormal cell and not a general normal population, having a high degree of sensitivity is critical for early detection.

Our studies provide evidence that the combination of *RARB* M4, *INK4a/ARF*, *PRB*, and *HIN-1* CpG island promoter methylation may predict non–BRCA1/2-associated mammary carcinogenesis and tumor progression. While there are limitations to our studies, i.e we are testing for promoter methylation in a small sample set (approximately 100 women and 40 women tested for BRCA1/2 mutations), our studies demonstrate a statistically significant association between promoter methylation events and BRCA1/2 mutation status. Validation studies are currently being performed in larger sample sets and studies are in progress to prospectively test the predictive value of these CpG island methylation markers in risk-stratifying women with mammary atypia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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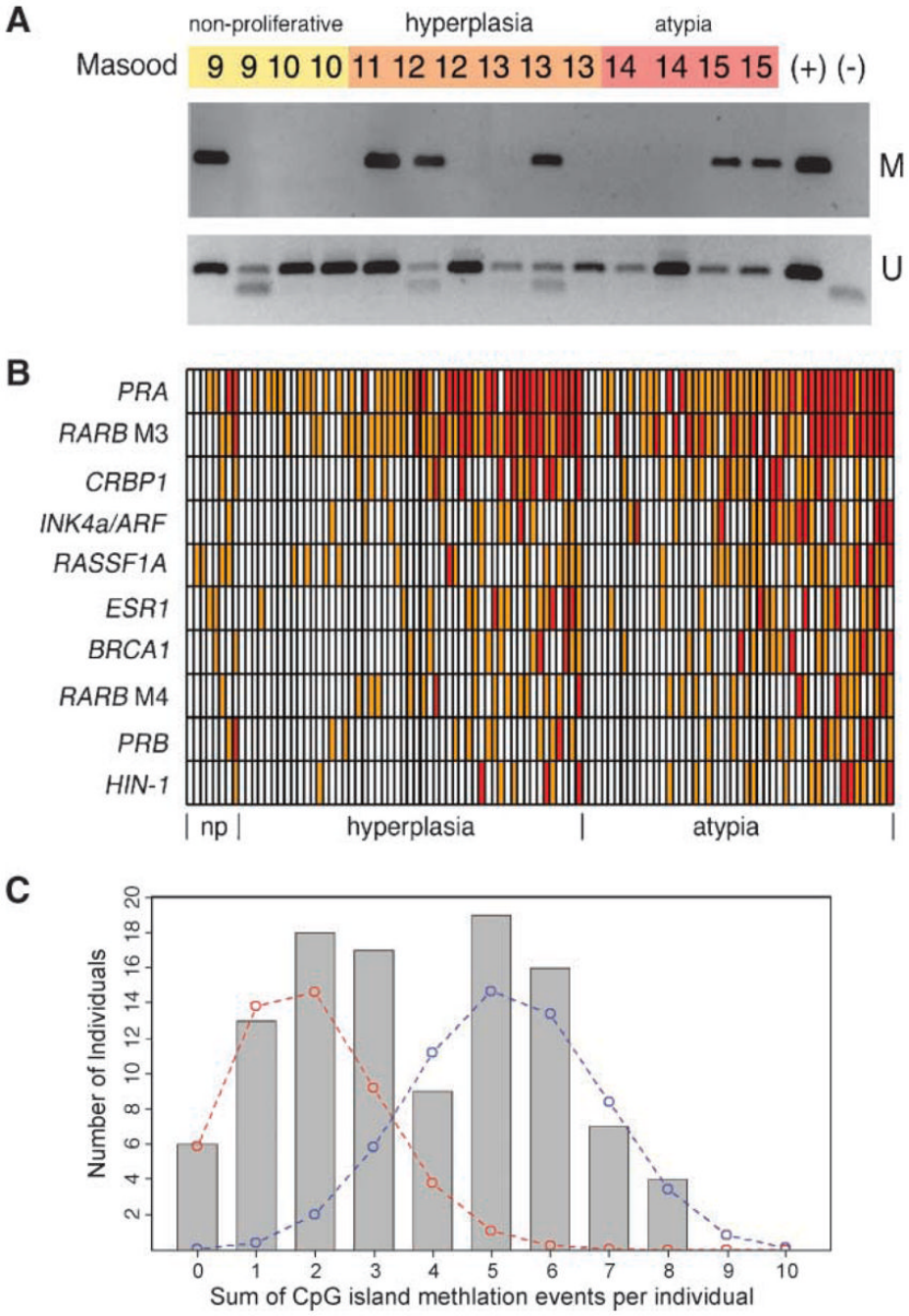

Figure 1.

Frequency and distribution of promoter methylation events in high-risk women. A total of 109 women were tested for methylation of 10 promoter targets: *RARB* (M3 and M4), *ESR1*, *INK4a/ARF*, *BRCA1*, *PRA*, *PRB*, *RASSF1A*, *HIN-1*, and *CRBP1*. **A.** Representative promoter methylation in RPFNA cytology. Methylation of the *ESR1* promoter in RPFNA obtained from 15 representative high-risk women with nonproliferative, hyperplastic, or atypical RPFNA. *M* and *U*, use of MSP primers to identify methylated and unmethylated *ESR1* promoter, respectively; (+), a methylated positive control in the methylated gels and the T47D breast cancer cell line in the unmethylated gels; (−), negative control. **B.** Promoter methylation events in women with nonproliferative (Masood score ≤10), hyperplastic (Masood score 11-13), and

atypical (Masood score ≥14) cytology. *Red column,* bilateral methylation; *orange column,* unilateral methylation; *white column,* no promoter methylation detected. Summary of three independent tests. *np,* nonproliferative. **C.** Sum of methylation events observed per individual versus the number of individuals. *Red* and *blue dotted lines,* theoretical fit of the data to a model of two binomial distributions.

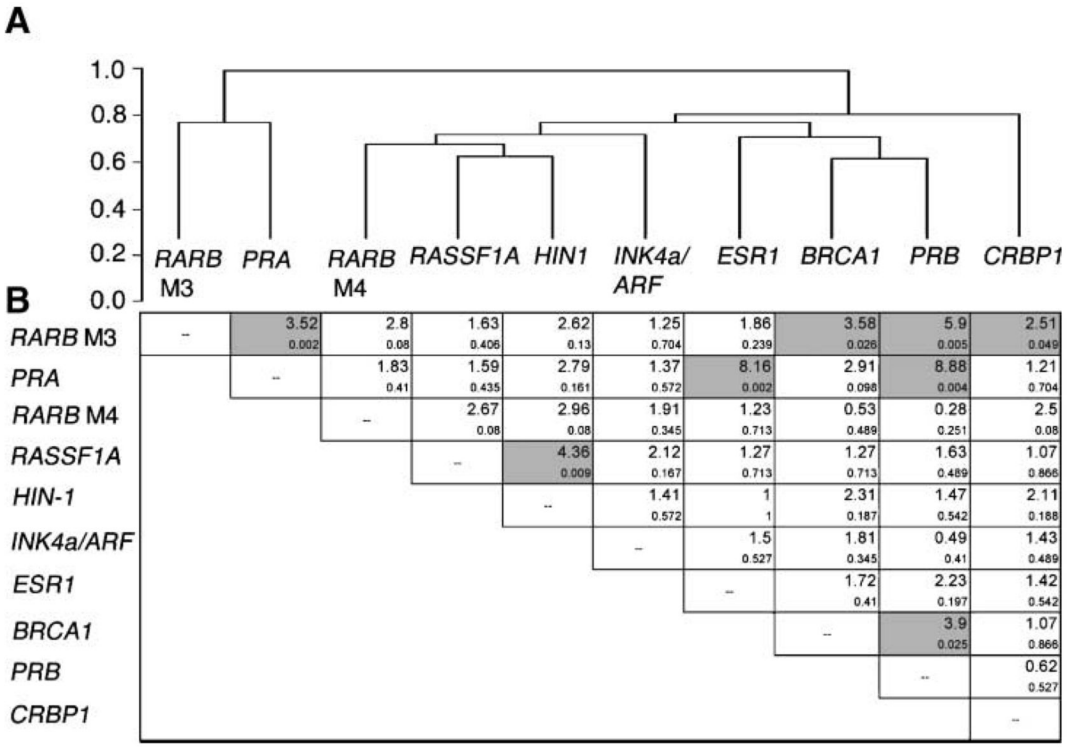

Figure 2.

Patterns of correlation among 10 promoter methylation markers from RPFNA cytology. Association between all pairwise combinations of 10 markers was examined in 189 RPFNA samples that had no missing data on promoter methylation. The 10 promoter targets are *RARB* (M3 and M4), *ESR1*, *INK4a/ARF*, *BRCA1*, *PRA*, *PRB*, *RASSF1A*, *HIN-1*, and *CRBP1*. **A.** Dendrogram for the agglomerative hierarchical clustering of promoter methylation states, showing patterns of similarity observed among markers. **B.** For all 45 pairs of markers, agreement was evaluated by Fisher's exact test. Results are estimated odds ratios and adjusted *P* values after correcting for the false discovery rate at the α = 0.05 level. *Gray*, significant pairwise correlations.

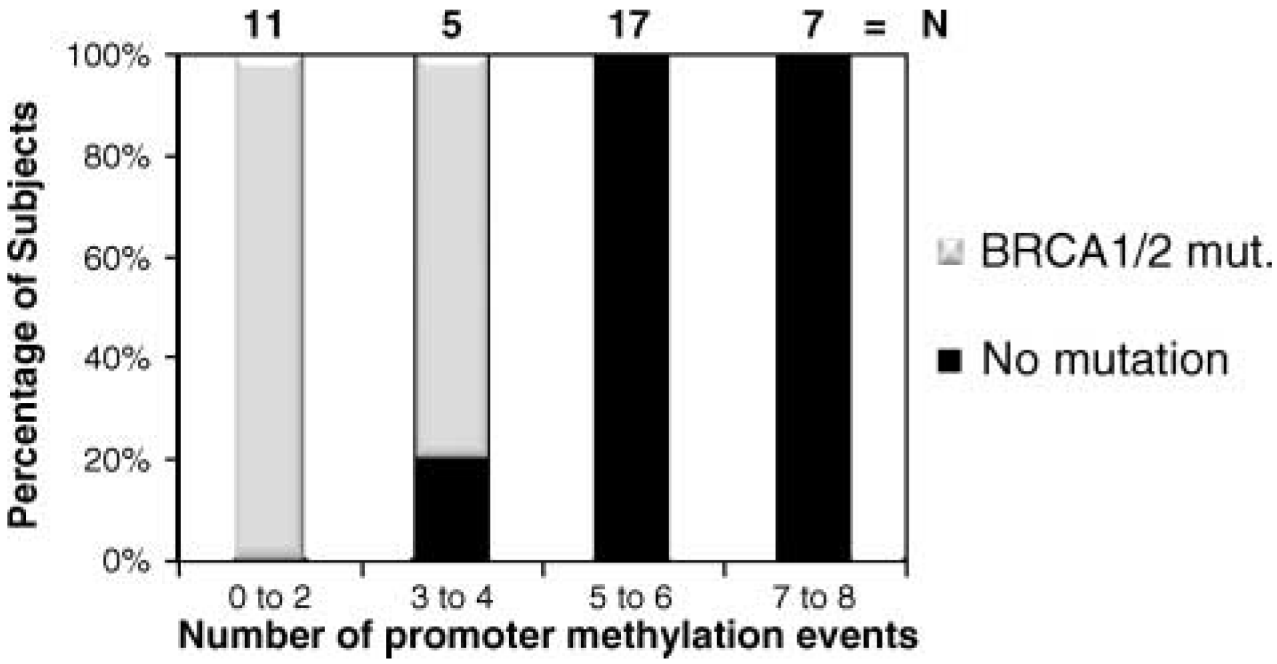

Figure 3.
Frequency of promoter methylation is inversely correlated with likelihood of carrying a BRCA1/2 mutation. Distribution and frequency of promoter methylation events in 25 women testing negative (*black*) and 15 women testing positive (*white*) for a BRCA1/2 mutation.

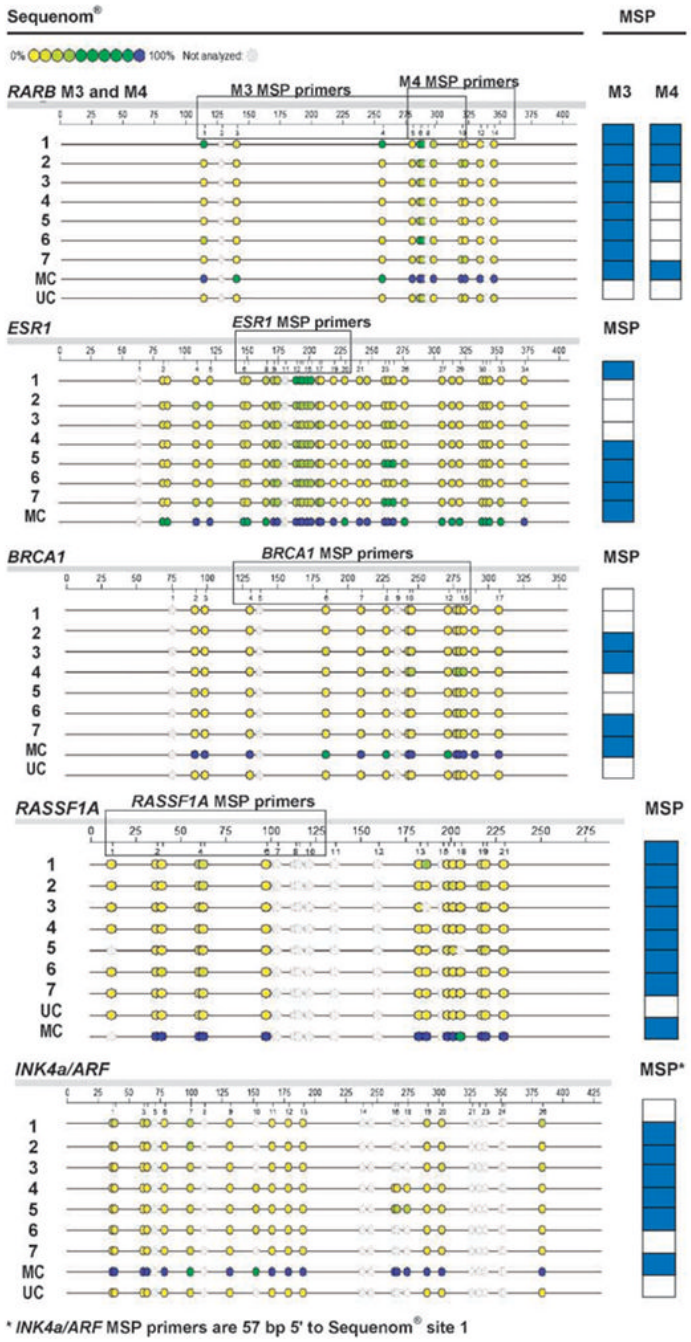

Figure 4.

Comparison of Sequenom methylation analysis with conventional MSP. Seven RPFNA samples as well as methylated and unmethylated control DNA samples were tested for methylation status by Sequenom and conventional MSP. Equal amounts (1 μg) of bisulfite-converted genomic DNA were used for each analysis. The sensitivity of Sequenom is 5%, whereas our conventional MSP assay is 0.01%. *MC*, methylated control; *UC*, unmethylated control. For MSP: *blue columns*, presence of methylation; *white columns*, absence of methylation.

Table 1

MSP primers and conditions

Gene	Orientation: primer sequence	bp	Anneal temperature (°C)	1 × Buffer (and additives)	CpG sites spanned
RARB2 M3	Forward: 5′-GGTTAGTAGTTCGGGTAGGGTTTATC Reverse: 5′-CCGAATCCTACCCCGACG	234	57	16.6 mmol/L $(NH_4)_2SO_4$, 67 mmol/L Tris (pH 9.1), 3 mmol/L $MgCl_2$	5
RARB2 U3	Forward: 5′-TTAGTAGTTTGGGTAGGGTTTATT Reverse: 5′-CCAAATCCTACCCCAACA	232	57	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.5), 4.5 mmol/L $MgCl_2$	
RARB2 M4	Forward: 5′-GTCGAGAACGCGAGCGATTC Reverse: 5′-CGACCAATCCAACCGAAACG	148	56	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 9), 3.5 mmol/L $MgCl_2$, 150 mmol/L 2-pyrrolidinone	8
RARB2 U4	Forward: 5′-GATGTTGAGAATGTGAGTGATTT Reverse: 5′-AACCAATCCAACCAAAACA	150	57	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.5), 4.5 mmol/L $MgCl_2$	
ESR1 M	Forward: 5′-GTGTATTTGGATAGTAGTAAGTTCGTC Reverse: 5′-CGTAAAAAAAACCGATCTAACCG	118	56	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 4 mmol/L $MgCl_2$, 100 mmol/L 2-pyrrolidinone	5
ESR1 U	Forward: 5′-GGTGTATTTGGATAGTAGTAAGTTTGT Reverse: 5′-CCATAAAAAAAACCAATCTAACCA	120	52	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.5), 4.5 mmol/L $MgCl_2$	
INK4a/ARF M	Forward: 5′-TTATTAGAGGGTGGGGCGGATCGC Reverse: 5′-GACCCCGAACCGCGACCGTAA	150	63	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 3.5 mmol/L $MgCl_2$, 100 mmol/L 2-pyrrolidinone	8
INK4a/ARF U	Forward: 5′-TTATTAGAGGGTGGGGTGGATTGT Reverse: 5′-CAACCCCAAACCACAACCATAA	151	57	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 9), 3.5 mmol/L $MgCl_2$, 275 mmol/L 2-pyrrolidinone	
BRCA1 M	Forward: 5′-GGTTAATTTAGAGTTTCGAGAGACG Reverse: 5′-TCAACGAACTCACGCCGCGCAATC	182	63	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 4 mmol/L $MgCl_2$, 100 mmol/L 2-pyrrolidinone	6
BRCA1 U	Forward: 5′-GGTTAATTTAGAGTTTTGAGAGATG Reverse: 5′-TCAACAAACTCACACCACACAATCA	182	63	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 9), 3.5 mmol/L $MgCl_2$	
PRA M	Forward: 5′-ACGGGTTATTTTTTTTTCG Reverse: 5′-TAAAATATACGCCCTCCACG	99	52	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.25), 4.5 mmol/L $MgCl_2$, 1× Q-solution (Qiagen)	4

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Gene	Orientation: primer sequence	bp	Anneal temperature (°C)	1 × Buffer (and additives)	CpG sites spanned
PRA U	Forward: 5′-ATGGGTTATTTTTTTTTTG Reverse: 5′-TAAAATATACACCCTCCACA	99	50	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 3 mmol/L $MgCl_2$, 250 mmol/L 2-pyrrolidinone	
PRB M	Forward: 5′-TGATTGTCGTTCGTAGTACG Reverse: 5′-CGACAATTTAATAACACGCG	200	59	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 3.5 mmol/L $MgCl_2$	6
PRB U	Forward: 5′-TGATTGTTGTTTGTAGTATG Reverse: 5′-CAACAATTTAATAACACACA	200	53	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.25), 4 mmol/L $MgCl_2$	
RASSF1A M	Forward: 5′-GGGTTTTGCGAGAGCGCG Reverse: 5′-GCTAACAAACGCGAACCG	167	60	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.5), 5.5 mmol/L $MgCl_2$	7
RASSF1A U	Forward: 5′-GGTTTTGTGAGAGTGTGTTTAG Reverse: 5′-CACTAACAAACACAAACCAAAC	167	58	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 7.75), 4.5 mmol/L $MgCl_2$	
HIN-1 M	Forward:5′-GTTTAGTTTTGAGGGGGGCGC Reverse: 5′-AACTTCCTACTACGACCGACG	163	60.5	15 mmol/L $(NH_4)_2SO_4$/ 60 mmol/L Tris (pH 8.25), 4 mmol/L $MgCl_2$	5
HIN-1 U	Forward:5′-ATTGTAAAGTGAAGGTGTGGGTT Reverse: 5′-CCAACTTCCTACTACAACCAACA	163	63	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8.25), 3.5 mmol/L $MgCl_2$	
CRBP1 M	Forward:5′-TTGGGAATTTAGTTGTCGTCGTTTC Reverse: 5′-AAACAACGACTACCGATACTACGCG	110	57	15 mmol/L $(NH_4)_2SO_4$, 60 mmol/L Tris (pH 8), 5 mmol/L $MgCl_2$, l× Q-solution (Qiagen)	7
CRBP1 U	Forward:5′-GTGTTGGGAATTTAGTTGTTGTTGTTTT Reverse: 5′-ACTACCAAAACAACAACTACCAATACTACA	110	58	15 mmol/L $(NFI_4)_2SO_4$, 60 mmol/L Tris (pH 7.75), 2 mmol/L $MgCl_2$, 0.02% DMSO	

Table 2

Patient characteristics for RPFNA

Women enrolled in study			109
Bilateral RPFNA			84
Unilateral RPFNA			25
RPFNA samples collected			193
Average age (range), y			47 (29-65)
Race, *n* (%)			
	Caucasian		98 (90)
	African American		11 (10)
Menopausal status, *n* (%)			
	Post/perimenopausal		44 (40)
	Premenopausal		65 (60)
Hormone replacement use, *n* (%)			
	Current		0 (0)
	Ever use		13 (12)
	Never use		96 (88)
Anti-estrogen therapy, *n* (%)			
	At the time of RPFNA		
		Tamoxifen	0 (0)
		Raloxifene	0 (0)
		Aromatase inhibitor	0 (0)
	Ever		
		Tamoxifen	1 (0.9)
		Raloxifene	1 (0.9)
		Aromatase inhibitor	0 (0)
Family history of breast cancer, *n* (%)			52 (48)
Prior abnormal biopsies, *n* (%)			
	Atypical ductal hyperplasia		13 (12)
	LCIS		4 (4)
	DCIS		9 (8)
	History of contralateral breast cancer		16 (15)
Tested for BRCA1/2 mutation, *n* (%)			40 (37)
	Tested positive		15 (14)
		BRCA1 mutation	13 (12)
		BRCA2 mutation	2 (2)
	Tested negative		25 (23)

Table 3

Frequency of RPFNA promoter methylation in RPFNA cytology

Marker		Frequency (%)
RARB M3		
	No	81 (41.97)
	Yes	112 (58.03)
	NA	0
RARB M4		
	No	158 (81.87)
	Yes	35 (18.13)
	NA	0
ESR1		
	No	152 (79.17)
	Yes	40 (20.83)
	NA	1
INK4a/ARF		
	No	150 (77.72)
	Yes	43 (22.28)
	NA	0
BRCA1		
	No	155 (81.58)
	Yes	35 (18.42)
	NA	3
PRA		
	No	65 (34.21)
	Yes	125 (65.79)
	NA	3
PRB		
	No	160 (84.21)
	Yes	30 (15.79)
	NA	3
RASSF1A		
	No	148 (77.89)
	Yes	42 (22.11)
	NA	3
HIN-1		
	No	161 (84.74)
	Yes	29 (15.26)
	NA	3
CRBP1		
	No	137 (72.11)
	Yes	53 (27.89)
	NA	3

NOTE: A total of 193 samples from 109 women were tested for methylation of 10 promoter targets: *RARB* (M3 and M4), *ESR1*, *INK4a/ARF*, *BRCA1*, *PRA*, *PRB*, *RASSF1A*, *HIN-1*, and *CRBP1*. No, lack of methylation; Yes, presence of methylation; NA, data not available.

Table 4

Association between promoter methylation and clinical variables

Marker	Frequency	Age	Masood score %	Premenopausal	Race (% Caucasian)	Body mass index	Gail	BRCA1PRO	BRCA2PRO
RARB M3									
No	33	49	13.0	48	91	24.1	2.5	0.00091	0.00073
Yes	76	45	13.0	67	89	23.3	2.1	0.0011	0.00062
P		0.10	0.19	0.067	0.82	0.50	0.88	0.98	0.69
RARB M4									
No	79	46	**13.0**	61	92	23.7	2.5	0.0013	0.00062
Yes	30	46.5	**13.5**	63	83	24.4	1.9	0.00080	0.00067
P		0.44	**0.051**	0.81	0.16	0.74	0.21	0.37	0.71
ESR1									
No	77	46	13.0	64	87	23.9	2.2	0.00091	0.00054
Yes	32	47.5	13.0	56	97	23.5	2.1	0.0028	0.0019
P		0.51	0.57	0.47	0.12	0.50	0.37	0.73	0.038
INK4a/ARF									
No	77	48	**13.0**	57	88	23.8	2.2	**0.0019**	**0.00076**
Yes	32	44	**14.0**	72	94	23.4	1.9	**0.00053**	**0.00033**
P		0.074	**0.042**	0.15	0.39	0.42	0.53	**0.010**	**0.031**
BRCA1									
No	80	47	13.0	61	89	24.0	2.4	0.00088	0.00058
Yes	29	46	14.0	62	93	23.1	2.1	0.0039	0.0012
P		0.56	0.32	0.94	0.50	0.16	0.64	0.35	0.34
PRA									
No	25	50	**12.0**	48	88	23.7	3.4	0.0013	0.00057
Yes	84	46	**13.0**	65	90	23.8	2.1	0.0011	0.00067
P		0.50	**0.032**	0.12	0.72	0.87	0.39	0.59	0.38
PRB									
No	84	47	13.0	**56**	89	23.9	2.2	0.00093	0.00064
Yes	25	44	14.0	**80**	92	22.8	2.1	0.0018	0.00062
P		0.11	0.90	**0.030**	0.69	0.22	0.99	0.77	0.93
RASSF1A									
No	71	46	13.0	62	89	23.8	2.2	0.0012	0.00064

Marker	Frequency	Age	Masood score %	Premenopausal	Race (% Caucasian)	Body mass index	Gail	BRCA1PRO	BRCA2PRO
Yes	38	47	14.0	61	92	23.5	2.2	0.0010	0.00058
P		0.41	0.20	0.88	0.58	0.60	0.39	0.64	0.78
HIN-1									
No	86	47	**13.0**	60	92	23.8	2.0	0.00093	0.00059
Yes	23	46	**14.0**	65	83	23.4	3.1	0.0020	0.00092
P		0.84	**0.044**	0.68	0.19	0.84	0.081	0.48	0.12
CRBP1									
No	69	48	13.0	61	91	**24.2**	2.2	0.0020	0.00081
Yes	40	44.5	14.0	63	88	**22.6**	2.1	0.00068	0.00046
P		0.33	0.087	0.87	0.53	**0.005**	0.94	0.31	0.28

NOTE: Median age, Masood score, body mass index, Gail model score, probability of a BRCA1/2 mutation (BRCAPRO scores), and the associated Wilcoxon rank-sum *P* value ($P \leq 0.05$ is statistically significant) for comparing the mean ranks of each covariate according to marker methylation status. Independently, the proportion of premenopausal and Caucasian women with a methylated marker is compared using Pearson's χ^2 test. Significant pairwise correlations are in bold. Yes, positive for methylation; No, negative for methylation.

Table 5

Frequency and distribution of promoter methylation events among women testing positive for BRCA1/2 mutations

Marker	No. (%) with methylation
PRA	8 (53)
RARB M3	6 (40)
CRBP1	2 (13)
ESR1	2 (13)
RASSF1A	2 (13)
BRCA1	2 (13)
PRB	1 (7)
RARB M4	1 (7)
INK4a/ARF	1 (7)
HIN-1	0 (0)

NOTE: The number (percentage) of women methylated for each marker of the 15 women testing positive for BRCA1/2.