Hypermethylation of the Breast Cancer–Associated Gene 1 Promoter Does Not Predict Cytologic Atypia or Correlate with Surrogate End Points of Breast Cancer Risk

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Abstract

Mutation of the breast cancer–associated gene 1 (BRCA1) plays an important role in familial breast cancer. Although hypermethylation of the BRCA1 promoter has been observed in sporadic breast cancer, its exact role in breast cancer initiation and association with breast cancer risk is unknown. The frequency of BRCA1 promoter hypermethylation was assessed in (a) 14 primary breast cancer biopsies and (b) the initial random periareolar fine-needle aspiration (RPFN A) cytologic samples obtained from 61 asymptomatic women who were at increased risk for breast cancer. BRCA1 promoter hypermethylation was assessed from nucleotide −150 to nucleotide +32 relative to the transcription start site. RPFN A specimens were stratified for cytologic atypia using the Masood cytology index. BRCA1 promoter hypermethylation was observed at similar frequency in nonproliferative (normal; Masood ≤10: 18%, 2 of 11), hyperplastic (Masood 11-13; 15%, 6 of 41), and atypical cytology (Masood 14-17: 22%, 4 of 18; P = 0.79). BRCA1 promoter hypermethylation was not associated with (a) family history of breast or ovarian cancer or (b) calculated Gail or BRCA PRO risk score. BRCA1 promoter hypermethylation was associated with (a) age (P = 0.028) and (b) the combined frequency of promoter hypermethylation of the retinoic acid receptor-β2 (RARB) gene, estrogen receptor-α (ESR1) gene, and p16 (INK4A) gene (P = 0.003). These observations show that BRCA1 promoter hypermethylation (a) is not associated with breast cancer risk as measured by mathematical risk models and (b) does not predict mammary atypia in RPFN A cytologic samples obtained from high-risk women. (Cancer Epidemiol Biomarkers Prev 2007;16(1):50–6)

Introduction

Since its discovery in 1994, numerous studies have shed light on the importance of the tumor suppressor breast cancer–associated gene 1 (BRCA1). BRCA1 mutations are highly penetrant and confer a high lifetime risk of breast and ovarian malignancy (1, 2). Positioned at 17q21, the locus has been extensively tested and linkage rates of 52% and 81% have been reported for families with prevalent cases of breast and ovarian cancer, respectively (1, 3). However, cases of BRCA1 germ-line mutation only represent 5% to 10% of all breast cancers (4), and the significance of BRCA1 in sporadic mammary carcinogenesis remains unclear.

BRCA1 is thought to regulate nuclear function and cell growth control via both DNA repair and transcription (5, 6). Work in knockout mice and BRCA1-deficient cell lines has implicated BRCA1 in acting to repair double-stranded breaks via homologous recombination and nonhomologous end-joining pathways (7, 8). Double-stranded breaks are recognized by BRCA1 via its association with the RAD50-MRE11-NBS1 protein complex (9). In addition, BRCA1 is implicated in the induction of stress-activated protein kinases as a result of cytotoxic stress. These stress-activated signaling pathways direct phosphorylation of cellular targets, leading to the activation of DNA repair and cell cycle arrest. Overexpression of BRCA1 induces the c-Jun NH2-terminal kinase pathway, leading to cell cycle arrest and apoptosis (10). BRCA1 also shows the ability to interact with signal transducers and activators of transcription 1 and sensitize cells to IFN-γ–mediated apoptosis (9, 11).

In the absence of BRCA1-dependent DNA repair, incorrect ligation of broken chromosomes may result in the loss or gain of function of genes that are important in cell growth control (12). As a caretaker gene expressed in numerous tissues, BRCA1 is therefore considered essential for the maintenance of genomic integrity. However, it is only in the breast and ovary that mutation carriers exhibit markedly increased risk for carcinogenesis. Additionally, BRCA1 germ-line mutations account for only 5% to 10% of breast cancers (4). Little is definitively known about the importance of BRCA1 loss in nonhereditary breast cancers; very few somatic mutations have been detected in sporadic cases (13, 14). Nevertheless, BRCA1 expression is lost or reduced in ~40% of sporadic breast cancers (15). Therefore, alternate mechanisms for loss must exist, and much work has focused on promoter hypermethylation and loss of heterozygosity (LOH) at the BRCA1 loci (17q12-q21).

LOH is proposed to be one mechanism for loss of BRCA1 expression (16, 17). A high rate of LOH at the BRCA1 locus was observed in one half of sporadic breast and ovarian cancers (18, 19). BRCA1 LOH has been reported in normal-appearing epithelium adjacent to sporadic breast lesions, advocating the late Helene Smith’s concept of a ‘field effect’ of increased risk for breast cancer (20). LOH at the BRCA1/BRCA2 loci is
observed in normal breast epithelium of BRCA1 and BRCA2 mutation carriers, suggesting that BRCA LOH is an early genetic event in nonmalignant cells of high-risk women (21).

Hypermethylation of the BRCA1 promoter is hypothesized to be a second mechanism for BRCA1 inactivation (22). Using varying modes of detection, breast cancer samples have yielded rates from 0% to 31% hypermethylated for BRCA1 (23–25). However, with reported proportions this low, it is clear that hypermethylation alone cannot account for the reduced BRCA1 expression seen in a majority of nonhereditary breast cancers (17, 25).

Random periareolar fine-needle aspiration (RPFNA) is a research technique developed to repeatedly sample mammary cells from the whole breast of asymptomatic high-risk women to assess both (a) breast cancer risk and (b) response to chemoprevention (26, 27). As indicated in previous studies, RPFNA has the advantage of being able to provide a “snapshot” of the whole breast, can be done successfully in a majority of high-risk women (72-85% cell yield), and has been validated in long-term chemoprevention cohorts (26-28). In high-risk women, the acceptance rate of RPFNA is high and ~80% of women who undergo initial RPFNA return for subsequent RPFNA (26, 27). The presence of detectable cellular atypia in a breast RPFNA specimen has been prospectively validated to confer a 5.6-fold increase in breast cancer risk in high-risk women (26). These studies underscore the use of RPFNA as a translational research tool, and as in our previous work, RPFNA can be used to couple cytologic analysis and methylation studies (28).

The contribution of BRCA1 promoter hypermethylation to early mammary carcinogenesis is currently unknown. As described in this study, we investigated the frequency and distribution of BRCA1 promoter hypermethylation in RPFNA cytologic specimens obtained from high-risk women. We do not observe an association between BRCA1 promoter hypermethylation and atypia or breast cancer risk as measured by mathematical risk models.

Materials and Methods

Informed Consent. The study was approved by the Human Subjects Committee and Institutional Review Board at Duke University Medical Center (RPFNA samples) or Ohio State University (tumor samples) in accordance with assurances filed with and approved by the Department of Health and Human Services.

Biopsy Tissue. Paraffin-embedded, fixed breast biopsy tissue was obtained from 14 subjects with ductal carcinoma in situ (DCIS) or stage I or II invasive breast cancer.

Eligibility. To be eligible for RPFNA, 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, lobular carcinoma in situ, or DCIS; (c) known BRCA1/BRCA2 mutation carrier; or (d) history of invasive breast cancer. In subjects with prior invasive cancer, DCIS, or radiation, only the contralateral breast was aspirated, as the cell yield from radiated breast tissue is uniformly poor. Women were required to be between 30 and 60 years of age, as women younger than 30 years have a low short-term risk of breast cancer and women older than 60 years often have involutional breasts that are unlikely to yield sufficient cells for analysis (26). Women younger than 30 years could only be aspirated if they were within 10 years of the age of onset for a first-degree relative. Women older than 55 years could only participate if they had moderate to high mammographic density or have a known high risk of breast cancer due to family history, personal history, or environmental exposure (including pesticide exposure and smoking).

Materials and Cell Culture Lines. DNA was extracted from primary breast tumors, and RPFNA as previously published; bisulfite treatment was as previously published (28).

DNA Extraction and Bisulfite Treatment. DNA was extracted from breast cancer cell lines, paraffin-embedded primary breast tumors, and RPFNA as previously published; bisulfite treatment was as previously published (28).

Methylation-Specific PCR. Previous work has elucidated appropriate methylation-specific PCR (MSP) primers encompassing the BRCA1 promoter (24, 36–38). The primer sequences used were as follows: M-5'GTTAATTATGGTTTGAGACG-3' (forward) and 5'TCAAGAATCTACGCGCCGATGC-3' (reverse); U-5'GTTAATTATGGTTTGAGACG-3' (forward) and 5'TCAAGAATCTACGCGCCGATGC-3' (reverse). All PCRs consisted of 50 ng bisulfite-treated DNA.

Mathematical Assessment of Breast Cancer Risk. Gail model and BRCA PRO risk assessments were done using the Breast Cancer Risk Assessment Tool3 and CancerGene4 software (29, 30). 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 (31). The BRCA PRO model calculates risk based on the probability of an individual carrying a mutation in the BRCA1 or BRCA2 genes using Bayesian methods to incorporate relevant family history, including second-degree relatives, of breast and/or ovarian cancers (32).

Random Periareolar Fine-Needle Aspiration. RPFNA was done as previously published (28). All investigators were trained to do RPFNA by C. Fabian. Cells from the right and left breast were processed separately, so as to obtain one specimen per aspirated breast. Epithelial cells were split into two samples, with half designated for cytology and half designated for DNA extraction.

Cytologic Assessment. Slides for cytology were prepared by filtration and Papanicolaou stained as described previously (26, 27). 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 (26, 27). Cells were classified qualitatively as nonproliferative, hyperplasia, or hyperplasia with atypia (33). Cytology preparations were also given a semiquantitative index score through evaluation by the Masood cytology index (34). As previously described, cells were given a score of 1 to 4 points for each of six morphologic characteristics that include cell arrangement, pleomorphism, number of myoepithelial cells, anisonucleosis, nucleoli, and chromatin clumping; the sum of these points computed the Masood score: ≤10, nonproliferative (normal); 11 to 13, hyperplasia; 14 to 17, atypia; >17, suspicious cytology (26, 34). Morphologic assessment, Masood cytology index scores, and cell count were assigned by a blinded, single dedicated pathologist (C.M.Z.; ref. 26).

Materials and Cell Culture Lines. Sodium bisulfite (Sigma, St. Louis, MO; A.C.S.) and hydroquinone (>99%; Sigma) were used under reduced lighting and stored in a desiccator. The T47D breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in supplemented αMEM (Life Technologies, Gaithersburg, MD; ref. 35).

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3 http://www.cancer.gov/bcrisktool/
4 http://www3.utsouthwestern.edu/cancergene/.
DNA, 1× PCR buffer, 250 μmol/L of each deoxynucleotide triphosphate, 200 mmol/L of each primer, and 2.5 unit of HotStar Taq polymerase (Qiagen, Chatsworth, CA) in 30 μL total volume. PCR buffers were individually optimized for the methylated and unmethylated programs. The 1× M buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 8.0), 4.0 mmol/L MgCl₂ and 100 mmol/L 2-pyridolidine (>99%; Fluka, Milwaukee, WI); the 1× U buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 9.0), and 3.5 mmol/L MgCl₂. Both methylated and unmethylated PCR programs consisted of 95°C for 5 min followed by 40 amplification cycles (94°C for 1 min, 63°C for 1 min, 72°C for 1 min) and a final extension of 72°C for 4 min. A GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) was used for all amplifications. PCR products were visualized on 1.5% ethidium bromide agarose gels using an Image Station 4400 (Kodak, Chicago, IL). Optimization with methylated primers was achieved using minute amounts (~50 pg) of CpGenome Universal Methylated DNA (Chemicon, Temecula, CA) to model RPFNA samples. To estimate PCR sensitivity, titrated experiments were done using known amounts of methylated, genomic positive control DNA (1 μg to 100 pg) spiked in unmethylated T47D genomic DNA for a total of 1 μg (24). Like all other breast cancer cell lines tested, T47D was consistently found to be unmethylated for BRCA1. Each sample was bisulfite treated and subjected to MSP as outlined above to determine the sensitivity of our method. Parallel reaction with CpGenome Universal Methylated DNA and without DNA template (i.e., water) served as positive and negative controls, respectively.

MSP conditions and primers for retinoic acid receptor-β2 (RAR-β2) at sites M3 and M4 were as already published (28); MSP primers for estrogen receptor-α as were published (39); conditions were identical to BRCA1 with the following exceptions: (a) annealing temperatures of 56°C and 52°C were used in the M and U programs, respectively, and (b) the 1× U buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 8.5), and 4.5 mmol/L MgCl₂. MSP primers for p16 were as published (40); conditions were identical to BRCA1 with the following exceptions: (a) annealing temperatures of 63°C and 57°C were used in the M and U programs, respectively; (b) the 1× M buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 8.0), 43.5 mmol/L MgCl₂, and 100 mmol/L 2-pyridolidine; and (c) the 1× U buffer consisted of 15 mmol/L (NH₄)₂SO₄, 60 mmol/L Tris (pH 9.0), 3.5 mmol/L MgCl₂, and 275 mmol/L 2-pyridylidinone.

Statistical Methods. The Wilcoxon rank sum test was used to compare BRCA1 promoter hypermethylation with age, Gail score, BRCAPRO score, Masood cytology index, RPFNA cell count, family history of cancer, and hypermethylation of four additional sites in the promoters of the RARB, ESR1, and p16 (INK4A) genes. The Spearman correlation coefficient was used to determine the association between cell count and Masood cytology index.

Results

Study Demographics. Fourteen primary paraffin-embedded breast cancer biopsy specimens from The Ohio State University Medical Center were tested for BRCA1 promoter hypermethylation. The study participants’ demographic and tumor stage information is listed in Table 1.

<table>
<thead>
<tr>
<th>Stage of breast cancer</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>Stage 0/DCIS</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Stage I</td>
<td>6 (43)</td>
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<tr>
<td>Stage II</td>
<td>6 (43)</td>
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The Wilcoxon rank sums test was used to compare BRCA1 promoter hypermethylation with age, Gail score, BRCAPRO score, Masood cytology index, RPFNA cell count, family history of cancer, and hypermethylation of four additional sites in the promoters of the RARB, ESR1, and p16 (INK4A) genes. The Spearman correlation coefficient was used to determine the association between cell count and Masood cytology index.

breast tissue, 51% (31 of 61) had unilateral RPFNA. Eighty-nine percent (54 of 61) of the women were Caucasian and 11% (7 of 61) were African American. Thirteen RPFNA subjects underwent BRCA1/BRCA2 mutation testing; BRCA1 and BRCA2 mutations were identified in two and four subjects, respectively. Thirty-four women were not considered appropriate for BRCA1/BRCA2 mutation testing based on family history; 14 women refused testing. The mean Masood cytology index of RPFNA specimens was 13. Of the 91 RPFNA samples that were collected, 21 had insufficient epithelial cells for cytologic testing, so 70 samples were submitted for full cytologic analysis.

Distribution of Masood Cytology in RPFNA. Seventy RPFNA specimens were stratified using the Masood cytology index. Sixteen percent (11 of 70) were nonproliferative (Masood ≤10), 56% (41 of 70) were hyperplastic (Masood 11-13), and 26% (18 of 70) were atypical (Masood 14-17).

Methylation Analysis. Hypermethylation from nucleotide –150 to nucleotide +32 of the BRCA1 promoter was tested using MSP (Fig. 1). This region includes the transcription start site and the beginning of exon 1A (41). A DNA sensitivity experiment was conducted with titrated amounts of a commercially available, positive control to model the RPFNA samples. MSP control assays detected 0.1% methylation (1 ng of positive control supplemented with unmethylated cell line for a total of 1 μg genomic DNA).

Incidence of BRCA1 Promoter Hypermethylation in Primary Breast Cancers. Hypermethylation of the BRCA1 promoter using MSP was tested in 14 paraffin-embedded primary breast cancer specimens. MSP analysis showed hypermethylation from nucleotide −150 to nucleotide +32 in 29% (4 of 14) of tumor samples (Fig. 2A).

Incidence of BRCA1 Promoter Hypermethylation in RPFNA. Hypermethylation of the BRCA1 promoter using MSP was tested in 91 RPFNA specimens. MSP analysis showed hypermethylation from nucleotide −150 to nucleotide +32 in 22% (20 of 91) of RPFNA samples (Fig. 2B). Of the 30 subjects who underwent bilateral RPFNA, 3% (1 of 30) exhibited bilateral BRCA1 promoter hypermethylation, 33% (10 of 30) exhibited unilateral BRCA1 promoter hypermethylation, and 63% (19 of 30) did not exhibit BRCA1 promoter hypermethylation in either breast. All included specimens

<table>
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<tr>
<th>Table 1. Characteristics of early-stage breast cancer patients</th>
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<tbody>
<tr>
<td>Women enrolled in study</td>
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<tr>
<td>Average age and range (y)</td>
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<tr>
<td>Race, n (%)</td>
</tr>
<tr>
<td>Caucasian</td>
</tr>
<tr>
<td>African American</td>
</tr>
<tr>
<td>Menopausal status, n (%)</td>
</tr>
<tr>
<td>Postmenopausal</td>
</tr>
<tr>
<td>Premenopausal</td>
</tr>
<tr>
<td>Type of tumor, n (%)</td>
</tr>
<tr>
<td>Invasive ductal</td>
</tr>
<tr>
<td>Invasive lobular</td>
</tr>
<tr>
<td>Mixed ductal/lobular</td>
</tr>
<tr>
<td>DCIS</td>
</tr>
<tr>
<td>Known BRCA1/BRCA2 mutation carriers, n (%)</td>
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Abbreviations: ER, estrogen receptor; PR, progesterone receptor.
exhibited strong unmethylated bands, confirming both the presence of DNA and the promoter sequence itself.

**Lack of Correlation of BRCA1 Promoter Hypermethylation in RPFNA and Masood Cytology Index Score.** RPFNA aspirates were stratified using the Masood cytology index (Fig. 3A). The distribution of BRCA1 promoter hypermethylation was reported as a function of increased cytologic abnormality. BRCA1 promoter hypermethylation was observed in 18% (2 of 11) of nonproliferative (normal; Masood ≤10), 15% (6 of 41) of hyperplastic (Masood 11-13), and 22% (4 of 18) of atypical cytology. The median Masood score did not differ between BRCA1 methylated and unmethylated samples (P = 0.79). The unmethylated group of 58 samples had a median Masood score of 13; the methylated group of 12 samples had a median Masood score of 12.

**Lack of Correlation of BRCA1 Promoter Hypermethylation in RPFNA and Cell Count.** The presence of BRCA1 promoter hypermethylation was compared with RPFNA cell count (Fig. 3B). The unmethylated group of 58 samples had a median cell count of 500 and the methylated group of 12 samples had a median cell count of 100; these two groups were not significantly different from each other (P = 0.50). Here and in previous studies, we observed a statistically significant association between Masood cytology index score and RPFNA cell count (P = 0.003; Fig. 3C; ref. 28).

**Lack of Correlation of BRCA1 Promoter Hypermethylation in RPFNA and Gail or BRCAPRO Model Risk Scores.** The associations between BRCA1 promoter hypermethylation and the (a) 5-year Gail model calculated risk and (b) BRCAPRO model risk score were tested. To do these analyses, subjects were considered hypermethylated for BRCA1 if promoter hypermethylation was detected in either (unilateral) or both (bilateral) RPFNA cytologic samples. Due to the limitations of the Gail model, only 52% (32 of 61) of our subjects could be assessed. As described previously (28), subjects (a) with history of DCIS, lobular carcinoma in situ, or invasive breast cancer; (b) younger than 35 years; and (c) who are African American could not be included in the Gail risk calculation. Despite the limitations of the Gail model in predicting risk in our cohort, there was no significant correlation between the frequency of BRCA1 promoter hypermethylation and the 5-year Gail risk score in the 32 subjects tested (P = 0.45). In contrast to the Gail model, risk calculation via BRCAPRO model was available for all subjects, except for one who was adopted. No correlation was observed between the presence of BRCA1 promoter hypermethylation and overall breast cancer risk calculated by the BRCAPRO model (P = 0.45 for BRCAPRO1 and P = 0.69 for BRCAPRO2).

**BRCA1 Promoter Hypermethylation in RPFNA Correlates with Age.** The presence of BRCA1 promoter hypermethylation (unilateral or bilateral) was compared with known clinical risk factors for breast cancer, including age, family history of cancer, and known BRCA1/BRCA2 mutation status. Only age showed an association with BRCA1 promoter hypermethylation (P = 0.028; Fig. 4A). The 69% (42 of 61) of subjects with unmethylated RPFNA samples had a median age of 45.5, whereas the 31% (19 of 61) of subjects with methylated samples had a median age of 49. No associations were found between BRCA1 promoter hypermethylation and family history of breast cancer (P = 0.70), premenopausal breast cancer (P = 0.80), or ovarian cancer (P = 0.95). The six subjects that were identified as BRCA1/BRCA2 carrier mutation carriers, no association was found with BRCA1 promoter hypermethylation; however, the low number of subjects limits any statistical analysis.

**Correlation of BRCA1 Promoter Hypermethylation in RPFNA with Other Epigenetic Markers.** A panel of three other genes implicated in mammary carcinogenesis (at four MSP sites) was evaluated for hypermethylation frequency. Promoter hypermethylation of RARB at M3 (nucleotide −51 to nucleotide +162) and M4 (nucleotide +104 to nucleotide +251; ref. 28), ESR1 (nucleotide +367 to nucleotide +494; ref. 39), and p16 (INK4A; nucleotide −150 to nucleotide +32; ref. 40) was evaluated in addition to BRCA1. BRCA1 promoter hypermethylation increased with higher cumulative frequency of other methylation markers (P = 0.003; Fig. 4B).

**Discussion**

Promoter hypermethylation is theorized to be an early event in carcinogenesis, and BRCA1 is thought to regulate DNA repair (5). BRCA1 promoter hypermethylation from nucleotide −567 to nucleotide +44 has been shown to reduce transcript expression (23, 25, 42–44). Thus far, BRCA1 promoter hypermethylation seems to be tumor specific and has been detected primarily in breast and ovarian cancers—the same tissues targeted by familial BRCA1 mutations (24, 44). Based on these observations, we hypothesized that promoter hypermethylation of BRCA1 could represent an alternative mechanism to mutation and promote early mammary carcinogenesis through loss of DNA repair function.
We tested the methylation status of the BRCA1 promoter in 14 early-stage primary breast tumors to initially test whether epigenetic changes in the BRCA1 gene are implicated in early mammary carcinogenesis. Although this initial determination is limited by the sample size, BRCA1 promoter hypermethylation occurred at a frequency of 29%, which is within the frequency range (11-31%) reported for sporadic breast cancers (23–25). Recently, BRCA1 promoter hypermethylation was reported in 9.1% of primary sporadic breast tumors using the same sets of unmethylated and methylated primers (45). The discrepancy in the hypermethylation frequency reported here and recent studies is potentially due to (a) size of the sampling population and (b) sensitivity and conditions of the MSP.

We then tested the association between BRCA1 promoter hypermethylation and early cytologic changes in 61 women at high risk for breast cancer. BRCA1 promoter hypermethylation was observed in 18% (2 of 11) of nonproliferative (normal; Masood V 10), 15% (6 of 41) of hyperplastic (Masood 11-13), and 22% (4 of 18) of atypical cytology. The median Masood score did not differ between BRCA1 methylated and unmethylated samples (P = 0.79; Fig. 3A). Our observations show that BRCA1 promoter hypermethylation does not predict cytologic atypia. BRCA1 promoter hypermethylation was associated with age (P = 0.028) and frequency of hypermethylation of three other genes [RARB, ESR1 and p16 (INK4A)] often implicated in mammary carcinogenesis (P = 0.003). However, besides age, no associations could be found between BRCA1 promoter hypermethylation and any clinical marker of risk or mathematical risk model, including Gail score (P = 0.45), the BRCAPRO model (P = 0.45 and P = 0.69 for 1 and 2, respectively), Masood cytology index (P = 0.79), and family history of breast cancer (P = 0.70), premenopausal breast cancer (P = 0.80), or ovarian cancer (P = 0.95). Due to the limitations of the Gail model, only 52% (32 of 61) of our subjects could be assessed and may alter results of our testing. In contrast, all subjects could be modeled by the BRCAPRO model.

Our observations show that BRCA1 promoter hypermethylation does not associate with family history of breast cancer or increased BRCAPRO model risk. It is attractive to hypothesize that promoter hypermethylation of BRCA1 explains the etiology of mutation-negative, familial breast and ovarian cancer. However, we do not observe an association between

Figure 2. BRCA1 promoter hypermethylation in breast cancer specimens and RPFNA. A. Hypermethylation of the BRCA1 promoter in 10 representative early-stage breast cancer specimens. B. Hypermethylation of the BRCA1 promoter in RPFNA obtained from eight representative high-risk women. Use of MSP primers to identify methylated (M) and unmethylated (U) BRCA1 promoter. +, hypermethylated positive control in the M gels and the T47D breast cancer cell line in the U gels; —, negative control.

Figure 3. No correlation between BRCA1 promoter hypermethylation and cytology. RPFNA aspirates were assessed for cell count and cytologic atypia using the Masood cytology index. The distribution of RPFNA specimens with BRCA1 promoter hypermethylation is depicted relative to Masood cytology index (A) and cell count (B). Masood cytology index is also reported relative to the total cell count of each sample (C).
BRCA1 promoter hypermethylation in RPFNA and mathematical or clinical predictors (except age) of breast cancer in our high-risk cohort. Other findings in the literature offer possible explanations for the discrepancy between the lack of BRCA1 promoter hypermethylation and both early cytologic abnormality and increased risk. In a recent report, breast cancer patients from high-risk families with negative BRCA1/2 genetic test results were found to have previously undetected genomic rearrangements in BRCA1, BRCA2, CHEK2, and p53, including within the promoters of these genes, using a more comprehensive analysis (46). Alternatively, reduced stability or dysregulated maintenance of steady-state levels of the BRCA1 protein may account for BRCA1 loss (47, 48).

Besides gender, age is the largest risk factor for developing breast cancer. Evidence is now accumulating that methylation changes may initiate in subpopulations of normal cells as a function of age and progressively increase during carcinogenesis (49). Age-related hypermethylation has been primarily studied in colon cancer, but several studies suggest it is a more universal phenomenon. Insulin-like growth factor-II, the myogenic differentiation gene, the paired box gene 6, and RAR-β1 all undergo age-related methylation in the colon; the hypermethylated in cancer gene 1 does so in the prostate and brain; and estrogen receptor-α and the tumor suppressor candidate gene 3 do so in the colon and liver (49). Here, we report the involvement of age-related promoter hypermethylation of BRCA1 in women at high risk for breast cancer.

In our previous work using the same cohort of high-risk women, promoter hypermethylation of the tumor suppressor gene RARB was found to associate with (a) cytologic atypia as measured by Masood cytology index (P = 0.0051) and (b) cell count (P = 0.003) in RPFNA (28). In other work, aberrant hypermethylation of the RAR-β2 promoter was associated with increased Gail risk (50). No correlation was found between RAR-β2 promoter hypermethylation and increasing age (P = 0.14). RAR-β2 promoter is frequently hypermethylated in both nonmalignant colonic epithelium and colorectal cancer. Frequent RAR-β2 promoter hypermethylation is observed in primary breast cancers, premalignant lesions, and surrounding morphologically normal-appearing epithelium (28, 51, 52). In contrast to colon cancer, hypermethylation of RAR-β2 in breast cancer is not age-related. It seems plausible that age-related hypermethylation is gene-specific and dependent on whether alternative mechanisms exist to protect epithelial cells from further DNA damage or de novo methylation. Given that no association was found between BRCA1 promoter hypermethylation and early cytologic atypia, yet an association was found with age, BRCA1 promoter hypermethylation does not seem to share the same initiating role as RAR-β2 suppression in early mammary carcinogenesis.

Hypermethylation in RPFNA samples with low-grade proliferative changes may represent an early event that becomes more prominent with age; these cells then set the stage for progression, undergoing further genetic and epigenetic changes that may confer a growth advantage. Thus, having the ability to repeatedly sample breast cells of women at high risk for breast cancer via RPFNA and test these cells for promoter hypermethylation patterns of multiple genes may begin to elucidate the functional significance of age-related methylation in predisposition to and progression of early mammary carcinogenesis. RPFNA may allow for closer monitoring of patients exhibiting high levels of age-related methylation and suggest which women would best benefit from chemoprevention.

The high-risk cohort used in this study reflects the heterogeneous nature of breast cancer and the diverse population of women with increased risk of developing breast cancer. This heterogeneity poses a challenge in clinical diagnosis of early breast disease, risk assessment, and appropriate course of treatments. Development of biomarkers that can provide useful information on the etiology of disease, risk assessment strategies, and early detection is a worthy goal. Given that (a) we could detect no correlation between the presence of BRCA1 promoter hypermethylation and increasing cytologic abnormality in RPFNA and (b) we and others find that the proportion of BRCA1 hypermethylation in mammary tumors is <30% (23–25, 45), BRCA1 promoter hypermethylation does not hold promise for risk-stratifying a majority of women at high risk for breast cancer.

References

Figure 4. Correlation between BRCA1 promoter hypermethylation and other risk factors. The distribution of RPFNA specimens with BRCA1 promoter hypermethylation is depicted relative to patient age (A) and the hypermethylation status of four other sites using MSP (B). The other sites include RARB (at M3 and M4; ref. 28), ESR1 (39), and p16 (INK4A; ref. 40).
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