Retinoic Acid Receptor-β2 Promoter Methylation in Random Periareolar Fine Needle Aspiration

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Abstract

Methylation of the retinoic acid receptor-β2 (RARβ2) P2 promoter is hypothesized to be an important mechanism for loss of RARβ2 function during early mammary carcinogenesis. The frequency of RARβ2 P2 methylation was tested in (a) 16 early stage breast cancers and (b) 67 random periareolar fine needle aspiration (RPFNA) samples obtained from 38 asymptomatic women who were at increased risk for breast cancer. Risk was defined as either (a) 5-year Gail risk calculation ≥1.7%; (b) prior biopsy exhibiting atypical hyperplasia, lobular carcinoma *in situ*, or ductal carcinoma *in situ*; or (c) known *BRCA1/2* mutation carrier. RARβ2 P2 promoter methylation was assessed at two regions, M3 (−51 to 162 bp) and M4 (104-251 bp). In early stage cancers, M4 methylation was observed in 11 of 16 (69%) cases; in RPFNA samples, methylation was present at M3 and M4 in 28 of 56 (50%) and 19 of 56 (38%) cases, respectively. RPFNAs were stratified for cytologic atypia using the Masood cytology index. The distribution of RARβ2 P2 promoter methylation was reported as a function of increased cytologic abnormality. Methylation at both M3 and M4 was observed in (a) 0 of 10 (0%) of RPFNAs with Masood scores of ≤10 (nonproliferative), (b) 3 of 20 (15%) with Masood scores of 11 to 12 (low-grade proliferative), (c) 3 of 10 (30%) with Masood scores of 13 (high-grade proliferative), and (d) 7 of 14 (50%) with Masood scores of 14 of 15 (atypia). Results from this study indicate that the RARβ2 P2 promoter is frequently methylated (69%) in primary breast cancers and shows a positive association with increasing cytologic abnormality in RPFNA. (Cancer Epidemiol Biomarkers Prev 2005;14(4):790–8)

Introduction

Recent studies suggest that breast cancer incidence may be substantially reduced in high-risk women by tamoxifen treatment and/or prophylactic mastectomy (1-3). Although these reports are encouraging, current prevention strategies are expensive and can be associated with significant side effects. Biomarkers are needed to accurately predict short-term breast cancer risk (a) so that women who are most likely to benefit from preventive therapy can be identified and (b) so that response to chemoprevention can be accurately assessed.

Retinoids are important mediators of growth and differentiation in normal human mammary epithelial cells (HMEC) and regulate the expression of many pharmacologic targets for prevention such as cyclooxygenase-2 (4-6). The majority of retinoid actions are mediated through specific nuclear retinoic acid receptors (RAR-α, RAR-β, and RAR-γ) and rexinoid X receptors (RXR-α, RXR-β, and RXR-γ). These nuclear receptors act as transcription regulators and establish genetic communication networks that are essential in regulating cell growth, differentiation, and apoptosis (4). The transcriptional activity of RARs and RXRs is primarily modulated through the formation of RAR/RXR heterodimers (4). These heterodimers have two distinct functions: (a) they modulate transcription initiation after binding to RAR elements in the promoter of target genes and (b) they promote ''cross-talk'' with other steroid signaling pathways, perhaps through promoting coactivator shifts.

RARβ2 is unique because it is (a) primarily expressed in epithelial cells and (b) positively regulated by retinoids and the RARβ2 P2 promoter RAR element (4). We have shown that RARβ2 is a tumor suppressor in breast cancer (7) and progressive loss of RARβ2 expression is observed during breast carcinogenesis (8, 9). Importantly, whereas retinoids and RARβ2 mediate growth arrest and differentiation in HMECs, restoration of RARβ2 function in breast cancer cells promotes apoptotic cell death (5, 10-12). Noncancerous epithelial cells adjacent to invasive breast cancer also exhibit markedly decreased RARβ2 mRNA expression (8, 9). This has led to the hypothesis that loss of RARβ2 expression may provide a local cellular environment (field effect) that promotes mammary carcinogenesis.

Tumorigenesis is thought to be a multistep process resulting from the accumulation of genetic losses and epigenetic changes. Epigenetic changes, mainly DNA methylation and modification of histones, are now recognized as playing a critical role in carcinogenesis (13). A multitude of studies using the candidate gene approach have established the importance of DNA hypermethylation in tumor suppressor gene silencing (13-24). Several important observations have been made. First, many tumor suppressor genes have been found to be hypermethylated in multiple tumor types. For example, *BRCA1* promoter hypermethylation has been observed in breast and ovarian cancer (16, 21). Second, DNA hypermethylation events occur early in carcinogenesis, which makes hypermethylation a potentially important marker of risk and a target for prevention. Third, evidence also suggests that hypermethylation of DNA repair genes may profoundly

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affect overall and disease-free survival in patients with malignancy (13).

Loss of RARβ2 function in mammary epithelial cells is hypothesized to be the result of both genetic and epigenetic events. Two mechanisms have been proposed: (a) loss of heterozygosity and (b) promoter hypermethylation (6, 8, 9, 14, 15). Loss of heterozygosity at the RARβ2 locus (*3p24*) is frequently observed in invasive breast cancers and is thought to be a late mechanism for loss of RARβ2 expression (6, 8). In contrast, RARβ2 P2 promoter methylation has been observed in dysplastic mammary epithelial cells and is thought to be an important early mechanism for loss of RARβ2 expression (9, 14, 15). Previous studies have determined that the RARβ2 P2 promoter is CpG rich and includes four regions of hypermethylation unique to breast cancer cells and absent in normal mammary epithelial cells (14). Whereas other methods of regulating RARβ2 transcription surely exist, studies in cell lines suggest a negative relationship between methylation and message expression (14). These findings are supported by studies where cancer cell lines with suppressed RARβ2 reexpress message when treated with a demethylating agent such as 5′-aza-deoxycytidine (14, 15).

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 (25, 26). RPFNA is distinct from diagnostic FNA: diagnostic FNA is a standard clinical technique used to evaluate a clinically identifiable breast mass, whereas breast RPFNA is analogous to a cervical Papanicolaou smear in its ability to obtain a representative sampling of cells from the entire breast of asymptomatic women. RPFNA has the advantage of being able to provide a ''snapshot'' of the whole breast, and unlike ductal lavage, (a) can be done successfully in a majority of high-risk women (72-85% cell yield for RPFNA versus 20-40% for ductal lavage) and (b) has been validated in long-term chemoprevention cohorts (25-27). A great strength of RPFNA is the willingness of high-risk women to undergo subsequent RPFNA; ~80% of women who undergo initial RPFNA undergo subsequent RPFNA (25, 26). Breast RPFNA has been successfully used to predict breast cancer risk in women at increased risk for breast cancer. The presence of any detectable cellular atypia in a breast RPFNA specimen is associated with a 5-fold increase in breast cancer risk in high-risk women (25). These observations validate the use of cellular atypia obtained by RPFNA as a surrogate marker of short-term breast cancer risk in high-risk populations.

The frequency of RARβ2 P2 promoter methylation and resulting loss of RARβ2 expression in breast RPFNA is currently unknown. RARβ2 P2 methylation is (a) frequently (74%) detected in fluid from mammary ducts containing ductal carcinoma *in situ* and invasive carcinomas and (b) observed in two of five (40%) atypical ductal lavage specimens (28, 29). Whereas these data are extremely limited (n = 5), they provide evidence for the feasibility of testing for RARβ2 P2 methylation in cytologic specimens. As described in this study, we show that RARβ2 P2 methylation (a) is observed in 69% of primary breast cancers and (b) correlates with the presence of increasing cytologic abnormality in RPFNA samples obtained in high-risk women.

Materials and Methods

Informed Consent. The study was approved by the Human Subjects Committee and Institutional Review Board at the Ohio State University (for biopsy assessment) and Duke University Medical Center (for RPFNA studies), 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 tested from subjects with stage I or II invasive breast cancer.

Eligibility. To be eligible for screening by 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*, ductal carcinoma *in situ* (DCIS); or (c) known *BRCA1/2* mutation carrier. 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. In general, 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 (25). Women younger than 30 years could only be aspirated if they were within 10 years of the age of onset in a first-degree relative. Women older than 55 years could only participate if they had prior evidence of generalized proliferative breast disease. All women were required to have a mammogram interpreted as ''not suspicious for breast cancer'' within 2 months of entry, plus a breast examination on the day of aspiration that was interpreted as normal or not sufficiently abnormal to warrant a diagnostic biopsy. Clinical variables evaluated included age, menopausal status, hormone and oral contraceptive use, parity, age of menarche and menopause, lactation history, family cancer history (including family history of breast, ovarian, colon, and prostate cancer), radiation exposure, and other environmental exposures.

RPFNA. RPFNA was done as previously published (25, 26). All investigators were trained to perform RPFNA by Carol Fabian. To control for hormonal effects on mammary cell proliferation, menstruating women were aspirated between days 1 and 12 of their cycle. The breast was anesthetized with 5 mL of 1% lidocaine, immediately adjacent to the areola, at ~3 and 9 o'clock positions. Eight to 10 aspirations were done per breast for random sampling of epithelial cells. After the aspiration, cold packs were applied to the breasts for 10 minutes, and both breasts were bound in kerlex gauze for 12 to 24 hours. Epithelial cells were pooled and placed in modified CytoLyt (Cytyc Co., Boxborough, MA) with 1% formalin for 24 hours. 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 (25, 26). 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 (25, 26). Cells were classified as nonproliferative, hyperplasia, or hyperplasia with atypia (30). Cytology preparations were also given a semiquantitative index score through evaluation by the Masood cytology index (25, 26). 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 (25, 26). Morphologic assessment, Masood cytology index scores, and cell count were assigned by a single dedicated pathologist from University of Kansas Medical Center (C.M.Z.) without knowledge of the subjects' clinical history.

Materials and Cell Culture Lines. Sodium bisulfite (Sigma, St. Louis, MO, A.C.S.) and hydroquinone (Sigma, >99%) were used under reduced lighting and stored in a dessicator. 2-Pyrrolidinone (>99%) was purchased from Fluka (Milwaukee, WI). HS578T and ZR751 cell lines were obtained from American

Type Culture Collection (Manassas, VA) and grown in supplemented αMEM (Life Technologies, Gaithersburg, MD) as previously described (5). HMEC-SR is a cell line derived from Human Papillomavirus E6-immortalized, normal HMEC strain AG11132 (M. Stampfer 172R/AA7; ref. 31). This cell strain was purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute, Camden, NJ) and grown in supplemented MEBM (Cambrex, Baltimore, MD) as previously described (11). E6-transduction is as previously described (32, 33).

DNA Extraction from Fixed Tissue. DNA was extracted from paraffin-embedded tissue using the Pico Pure DNA Extraction Kit (Arcturus, Mountain View, CA) according to manufacturer's instructions. The DNA was purified with a phenol-chloroform extraction, ethanol precipitated, and resuspended in 10 mmol/L Tris (pH 7.5). Samples were stored at −80°C.

DNA Extraction from RPFNA. The RPFNA samples were washed with unmodified CytoLyt to eliminate RBCs. The cells were treated with proteinase K digestion buffer [50 mmol/L Tris (pH 8.1), 1 mmol/L EDTA, 0.5% Tween 20, and 0.1 mg/mL proteinase K] and incubated overnight at 40°C (34). The proteinase K was inactivated by incubation at 95°C for 10 minutes, the samples were spun, and the supernatant was collected and stored at −80°C.

Confirmation of Genomic Integrity. To confirm the integrity of the extracted genomic DNA from fixed tissue, PCR analysis was used to detect β-actin. PCR reactions consisted of 50 ng DNA, 1× PCR buffer (Roche, Nutley, NJ), 250 μmol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 2.5 units of Taq polymerase (Roche) in 30 μL total volume. Amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) as follows: initial 95°C for 5 minutes followed by 40 amplification cycles (94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute) and a final extension of 72°C for 4 minutes. Primer sequences were as follows: 5′-CCCGCTACCTCTTCTGGTG-3′ (sense) and 5′-GGGGTGTTGAAGGTCTCAAA-3′ (antisense).

Bisulfite Treatment. Extracted DNA from both RPFNA and fixed tissue was sodium bisulfite treated following the protocol of Grunau et al., with some modifications (35). Treatments on positive and negative controls were done simultaneously. Commercially available, fully methylated human DNA (Amersham, Arlington Heights, IL) was used as a positive control. Briefly, 1 μg of genomic DNA was denatured with 3 mol/L NaOH for 20 minutes at 42°C followed by deanimation in saturated sodium bisulfite and 10 mmol/L hydroquinone solution (pH 5.0) for 4 hours at 55°C in the dark. The samples were desalted using the Wizard DNA Clean-Up System (Promega, Madison, WI) according to the manufacturer's protocol. The DNA was then desulfonated in 3 mol/L NaOH for 20 minutes at 37°C, ethanol precipitated, and resuspended in 1 mmol/L Tris (pH 8.0) for storage in aliquots at −20°C.

Methylation-specific PCR. This assay takes advantage of discriminatory primers for methylated and unmethylated DNA, as the primers bind or do not bind depending on methylation status. Previous work has elucidated four CpG regions where methylation is known to occur upstream from the *RARβ2* gene; region 3 (M3) includes the RAR element and TATAA box and region 4 (M4) includes the 5′ end of the transcribed message (14). Region 4 was first studied in the fixed tumor samples, whereas both regions 3 and 4 were investigated in the RPFNA samples. All PCR reactions consisted of 50 ng DNA, 1× PCR buffer (see Table 2), 250 μmol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 2.5 units of HotStar Taq polymerase (Qiagen, Chatsworth, CA) in 30 μL total volume. Each PCR thermal cycle consisted of 95°C for 5 minutes followed by 40 amplification cycles (94°C for 1 minute, annealing temperature for 1 minute, and 72°C for 1 minute) and a final extension of 72°C for 4 minutes. See Table 1 for primer sequences and annealing temperatures (14). A GeneAmp PCR System 9700 (Applied Biosystems) was used for all amplifications. PCR products were visualized on 1.5% ethidium bromide agarose gels using an Image Station 440 (Kodak, Chicago, IL). Optimization with methylated primers was achieved using minute amounts of methylated positive control (~50 pg) to model RPFNA samples.

Methylation-specific PCR Sensitivity Experiment. Previous studies have investigated the methylation status of breast cancer cell lines at both regions 3 and 4 (14, 15). It was confirmed that cells of the HMEC-SR line were unmethylated at region 3, HS578T cells were unmethylated at region 4, and ZR751 cells were methylated at both regions. As an estimate of PCR sensitivity, two experiments were set up where known amounts of methylated cells were titrated in unmethylated cells of each negative type. For the purposes of testing, the RPFNA procedure was estimated to yield an average of two million epithelial cells. Thus, titrated amounts of ZR751 cells (0-100,000 cells) were used to spike two million cells of each negative type. Each sample was DNA extracted, bisulfite treated, and subjected to PCR as outlined above to determine the sensitivity of our method at each region.

Table 1. MS-PCR primer sequences and reaction conditions

	Sequences	1× Buffer (and additives)	Annealing temperature (°C)
M3	S 5′-GGTTAGTAGTTCGGGTAGGGTTTATC-3′, AS 5′-CCGAATCCTACCCCGACG-3′	16.6 mmol/L $(NH_4)_2SO_4$ 67 mmol/L Tris (pH 9.1) 3.0 mmol/L $MgCl_2$	57
U3	S 5′-TTAGTAGTTTGGGTAGGGTTTATT-3′, AS 5′-CCAAATCCTACCCCAACA-3′	15 mmol/L $(NH_4)_2SO_4$ 60 mmol/L Tris (pH 8.5) 4.5 mmol/L $MgCl_2$	57
M4	S 5′-GTCGAGAACGCGAGCGATTC-3′, AS 5′-CGACCAATCCAACCGAAACG-3′	15 mmol/L $(NH_4)_2SO_4$ 60 mmol/L Tris (pH 9.0) 3.5 mmol/L $MgCl_2$ 150 mmol/L 2-pyrrolidinone	55
U4	S 5′-GATGTTGAGAATGTGAGTGATTT-3′, AS 5′-AACCAATCCAACCAAAACA-3′	15 mmol/L $(NH_4)_2SO_4$ 60 mmol/L Tris (pH 8.5) 4.5 mmol/L $MgCl_2$	57

Abbreviations: S, sense; AS, antisense.

Table 2. Patient characteristics of early-stage breast cancer

Women enrolled in study	16
No. biopsy samples taken	17
	n = 16 (%)
Average age and range (y)	57 (34-82)
Race	
Caucasian	14 (88)
African American	2 (12)
Menopausal status	
Postmenopausal	11 (69)
Premenopausal	5 (31)
Stage of breast cancer	
Stage 0/DCIS	1 (6)
Stage I	8 (50)
Stage II	7 (44)
Average tumor size and range (cm)	3.2 (0.5-5.0)
Type of tumor	
Invasive ductal	12 (75)
Invasive lobular	1 (6)
Mixed ductal/lobular	2 (12)
DCIS	1 (6)
Tumor receptor status	
ER^+	11 (69)
PR^+	8 (50)
Lymph node–positive disease	5 (31)

Statistical Methods. The Wilcoxon rank sums test was used to compare the median Masood score with M3 methylation, M4 methylation, and a combination of both M3 and M4 methylation. Median cell counts and M3 or M4 methylation were also compared using the Wilcoxon rank sums test. The Spearman correlation coefficient was used to determine the association between cell count and Masood score.

Results

Study Demographics. Sixteen women with early stage breast cancer (stages 0-II) were enrolled at Ohio State University from April 1999 to December 1999. Six percent had stage 0 breast cancer (DCIS), 50% had stage I breast cancer, and 44% had stage II breast cancer. The average tumor size was 3.2 cm (range, 0.5-5 cm). Thirty-one percent of the women had lymph node positive disease. Sixty-nine percent of the tumors were estrogen receptor positive and 50% were progesterone receptor positive. The mean age of the women was 57 years old (range, 34-82 years). Eighty-eight percent of the women were Caucasian and 12% were African American. Sixty-nine percent of the women were postmenopausal. A summary of clinical characteristics of subjects with early stage breast cancers whose primary breast biopsy specimens were tested for RARβ2 P2 promoter methylation are listed in Table 2.

Thirty-eight women underwent RPFNA at Duke University Medical Center from March 2003 to March 2004. Clinical characteristics of subjects undergoing RPFNA are listed in Table 3. The mean age was 46 years (range, 29-64 years). Forty-seven percent of the women were either perimenopausal or postmenopausal; 53% were premenopausal. Seventy-four

Table 3. Clinical characteristics of patients undergoing RPFNA

A. Patient characteristics for RPFNA

Women enrolled in study	38
Bilateral RPFNA	28
Unilateral RPFNA	10
RPFNA samples collected	66
No. RPFNAs with insufficient epithelial cell count	10
No. RPFNAs submitted for analysis	56
	n = 38 (%)
Average age and range (y)	46 (29-64)
Race	
Caucasian	33 (87)
African American	5 (13)
Menopausal status	
Postmenopausal	18 (47)
Premenopausal/Perimenopausal	20 (53)
Hormone replacement use	
Current	2 (5)
Ever use	9 (24)
Never use	27 (71)
Antiestrogen therapy (at the time of RPFNA)	
Tamoxifen	2 (5)
Raloxifene	1 (3)
Aromatase inhibitor	2 (5)
Family history of breast cancer	17 (45)
Prior abnormal biopsies	
LCIS	1 (3)
DCIS	5 (13)
ADH	10 (26)
History of contralateral breast cancer	5 (13)

B. Characteristics of patients on prevention therapy at time of RPFNA

RPFNA sampling	Agent	Duration	Masood	Cell count	M3/M4 methylation
Unilateral	Tamoxifen	2 wk	14	500	+/+
Bilateral (L)	Raloxifene	2 y	10	<10	−/−
Bilateral (R)			10	10	−/−
Unilateral	Tamoxifen	2 wk	15	1,000	+/−
Unilateral	Aromatase inhibitor	1 y	NA	NA	−/−
Unilateral	Aromatase inhibitor	1 y	NA	NA	−/−

percent of the women had bilateral RPFNA (28 of 38), as RPFNA was not done on breast tissue with a history of invasive cancer or DCIS. Patients who underwent bilateral RPFNA contributed two separate samples for purposes of analysis. Eighty-seven percent (33 of 38) of the women were Caucasian and 13% (5 of 38) were African American. Five percent (2 of 38) of the women were currently on hormone replacement at the time of RPFNA. Twenty-four percent (9 of 38) of the women had been on estrogen replacement in the past and had a mean of 9 years of exposure (range, 1-25 years). Thirteen percent (5 of 38) received tamoxifen, raloxifene, or an aromatase inhibitor at time of RPFNA. The mean Masood cytology index was 12. Table 3B provides patient characteristics, duration of therapy, Masood score, and methylation for this subset of patients. Of the 66 RPFNA samples collected, 10 had insufficient epithelial cells for cytologic testing (one cell cluster with >10 epithelial cells); thus, 56 samples were submitted for full cytopathology analysis. Of the samples that contained insufficient cell counts for cytologic analysis, six samples both yielded methylation information and were positive for β-actin by PCR amplification (data not shown). Of the RPFNA samples, 33% (18 of 56) were from participants with a prior abnormal biopsy (20% ADH, 11% DCIS, 2% lobular carcinoma *in situ*), 8 of 56 had history of contralateral breast cancer, 20 of 56 had strong family history, and 2 of 56 were *BRCA2* mutation carriers.

Methylation Analysis. In this study, two known potential hypermethylation regions of the RARβ2 P2 promoter were examined in mammary epithelial cells. These regions are detailed in Fig. 1. Region 3 (M3) contains the RAR element (nucleotides, nt −52 to −36), TATAA box (nt −28 to −24), and the transcription start region; methylation region 4 (M4) contains an Sp1 element (nt 230-235; ref. 14). In the RPFNA samples, either only an unmethylated band or both methylated and unmethylated bands were observed. The presence of unmethylated RARβ2 promoter sequences confirmed the integrity of the DNA in all samples and was expected given the global nature of the sampling. A cellular sensitivity experiment was conducted with titrated amounts of a cell line known to be methylated to model the RPFNA samples. In this heterogeneous environment, the Methylation-specific PCR (MS-PCR) assay was sensitive enough to detect 0.005% methylation at region 3 (100 ZR751 cells in two million HMEC-SR cells) and 0.05% methylation at region 4 (1,000 ZR751 cells in two million HS578T cells). Similarly, MS-PCR was able to detect ~5 and ~50 pg, respectively, of a bisulfite-treated methylated positive control submitted to the same experimental conditions as the RPFNA samples.

Incidence of RARβ2 P2 Promoter Methylation in Primary Breast Cancers. The frequency of RARβ2 P2 promoter methylation was tested in primary breast cancer samples at methylation region 4 (M4; Fig. 2B). PCR amplification of β-actin was used to confirm DNA integrity (data not shown). MS-PCR analysis of these 16 samples showed methylation in 69% of primary breast cancers at region M4. These results are similar to previously published reports (14).

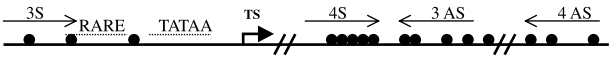

Figure 1. MS-PCR targets. MS-PCR primers were designed to amplify regions of known methylation regions in the RARβ2 P2 promoter (14). M3 region (methylated nt −51 to 162; unmethylated nt −49 to 162) includes the RARE, TATAA box, and transcription start region; M4 region (methylated nt 104-251; unmethylated nt 101-250) contains an Sp1 element. •, methylated CpGs were previously identified by sequencing of cell lines and primary tumor samples (14, 15).

Incidence of RARβ2 P2 Promoter Methylation in RPFNA. Methylation-specific PCR at the M3 and M4 regions was tested in 66 RPFNA specimens. Fifty-six of 66 samples (85%) had sufficient cellular material for cytologic analysis. MS-PCR analysis of these 56 samples showed methylation in 50% (28 of 56) of RPFNA samples at region M3 and 38% (21 of 56) of RPFNA samples at region M4. All included samples exhibited strong unmethylated bands at both regions, confirming both the presence of DNA and the promoter sequence itself (Fig. 2C). Sixty-four percent (36 of 56) of RPFNA samples showed methylation at either M3 or M4; 23% (13 of 56) showed methylation at both M3 and M4. Twenty-eight of the subjects had bilateral RPFNAs; 12 paired samples did not have sufficient cellular material so only 16 paired samples were analyzed. Of these samples, 5 of 16 (31%) showed methylation in RPFNA bilaterally, 7 of 16 (44%) showed methylation in RPFNA unilaterally, and 4 of 16 (25%) showed the absence of methylation in either breast.

Correlation of RARβ2 P2 Promoter Methylation in RPFNA with Masood Cytology Index Scores. RPFNA aspirates were stratified for cytologic atypia using the Masood cytology index. The distribution of RARβ2 P2 promoter methylation was reported as a function of increased cytologic abnormality. Figure 3A shows the number of samples with M3 region methylation for each Masood score. As the Masood score increased, the percentage of samples with M3 region methylation increased. Importantly, no sample with a Masood score of ≤10 exhibited RARβ2 P2 promoter methylation at either the M3 or M4 region. The 28 samples without M3 region methylation had a median Masood score of 11, whereas the 29 samples with M3 region methylation had a median Masood score of 13. There was a significant difference between the two groups (P = 0.0018). Figure 3B shows the number of samples with M4 region methylation for each Masood score. The 35 samples without M4 region methylation had a median score of 12, whereas the 20 samples with M4 region methylation had a median Masood score of 13.5 (P = 0.0002). Figure 3C shows the presence of both M3 and M4 region methylation for each Masood score. The 42 samples without methylation at both regions had a median Masood score of 12. The 13 samples with both M3 and M4 region methylation had a median Masood score of 14 (P = 0.0051).

Correlation of RARβ2 P2 Promoter Methylation in RPFNA with Cell Count. The presence of RARβ2 P2 promoter methylation was compared with total cell count of each corresponding RPFNA slide (Fig. 4A and B). The 22 samples without M3 or M4 region methylation had a median cell count of 10, whereas the 34 samples with either M3 or M4 region methylation had a median cell count of 300 (P = 0.003).

Correlation of Masood Score with Cell Count. RPFNA Masood scores were compared with the total cell count of each sample (Fig. 4C). The Spearman correlation coefficient is 0.67 and indicates a significant correlation between the cell count and the Masood score (P < 0.0001).

Discussion

Tumorigenesis is hypothesized to be a multistep process resulting from the accumulation of genetic losses and epigenetic changes. Epigenetic changes, mainly DNA methylation and modification of histones, are now recognized as playing a critical role in mammary carcinogenesis (13, 36). To better define the role of promoter hypermethylation in early mammary carcinogenesis, we prospectively tested (a) the frequency of RARβ2 P2 promoter hypermethylation in RFPNA specimens obtained from women at high-risk for breast cancer and (b) whether the presence of RARβ2 P2 promoter hypermethylation correlates with the presence of early cytologic changes.

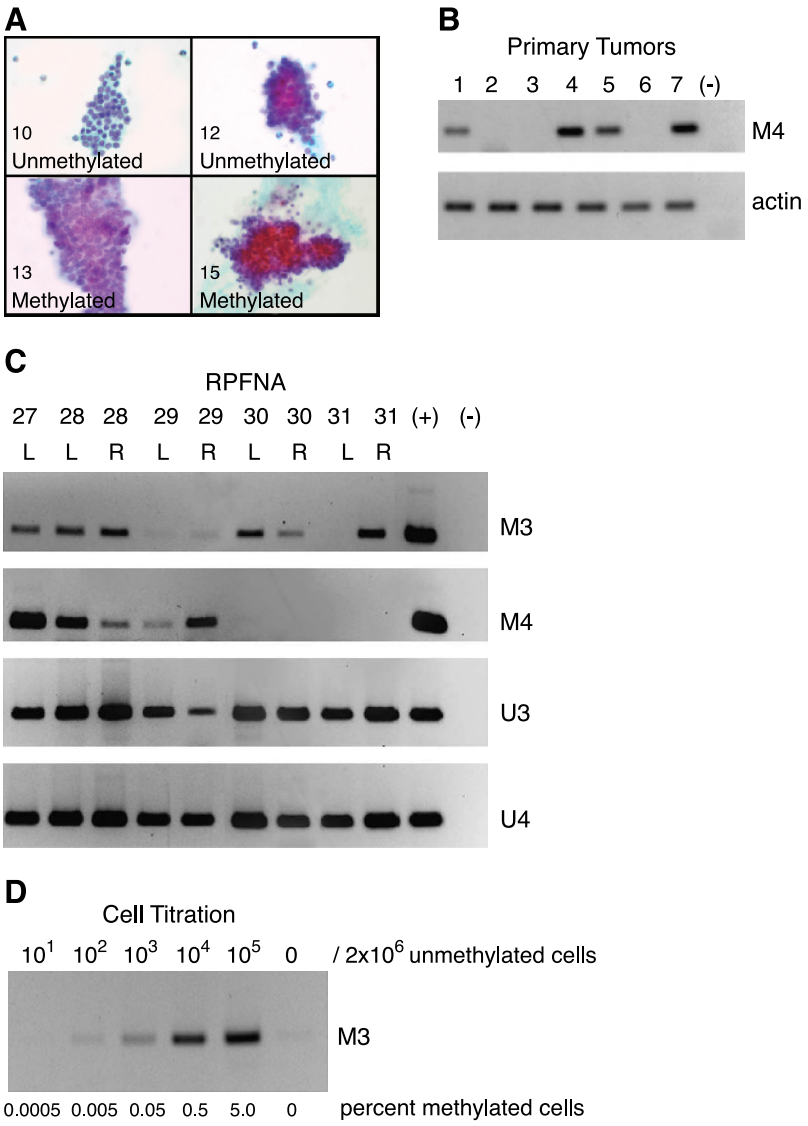

Figure 2. RPFNA cytology and methylation of RARβ2 P2 promoter. **A.** Representative RPFNA specimens in high-risk women. *Numeric value,* Masood cytology index score for this specimen. Presence of RARβ2 P2 promoter methylation at either the M3 or M4 region (*Methylated*). If neither region is methylated, the specimen is labeled *Unmethylated*. **B.** Hypermethylation of RARβ2 P2 promoter M4 region in early-stage breast cancer specimens. To confirm the integrity of the extracted genomic DNA, PCR analysis was used to detect β-actin (*actin*). **C.** Hypermethylation of RARβ2 P2 promoter M3 and M4 regions in RPFNA obtained from five representative high-risk women. **D.** Hypermethylation of RARβ2 P2 promoter M3 region in cellular sensitivity experiment to measure lowest threshold of methylation detection in a sea of unmethylated DNA, as explained in Materials and Methods. *M3* and *M4,* use of primers to identify methylated RARβ2 P2 regions 3 and 4, respectively. *U3* and *U4,* use of primers to identify unmethylated RARβ2 P2 regions 3 and 4, respectively. Methylated positive control in the M3 and M4 gels, HMEC-SR in the U3 gel, and HS578T in the U4 gel (+). Negative control (−).

In 1996, the late Helene Smith proposed a model of mammary carcinogenesis where breast cancer developed in a ''high-risk epithelial field'' in which some of the genetic and epigenetic aberrations found in cancer may also be present in the morphologically normal surrounding epithelium (8). Loss of heterozygosity of RARβ2 has been detected in normal epithelium adjacent to breast cancer but not in distal epithelium, supporting a ''field effect'' of increased risk (8). Yet, it has been found that loss of heterozygosity cannot fully account for the frequent loss of RARβ2 expression in breast cancer (37). Furthermore, the presence of methylation has been shown to correlate between tumor and the apparently normal adjacent tissue. For example, RARβ2 P2 promoter methylation has been detected in normal adjacent tissue of head and neck squamous cell carcinoma tumors where RARβ2 P2 promoter methylation was also present; RARβ2 P2 methylation was not detected in normal epithelium near unmethylated head and neck squamous cell carcinoma (38). Taken together, an early epigenetic change such as methylation may contribute to this ''field effect'' seen in breast cancer, and RARβ2 P2 promoter methylation occurs early enough along this progression to potentially serve as an effective biomarker.

In this study, we observe that RPFNA specimens obtained in women at high risk for breast cancer exhibit (a) a high frequency of methylation at the M3 and M4 regions of the RARβ2 P2 promoter and (b) a positive correlation between the presence of RARβ2 P2 promoter methylation and increasing Masood cytologic abnormalities (Fig. 3). RARβ2 P2 promoter methylation was also observed in 69% of primary low-risk breast cancers and, importantly, was not observed in 10 nonproliferative, cytologically normal RPFNA specimens (Masood index, ≤10). The frequency of RARβ2 P2 promoter methylation at the M3 region was unexpectedly high in RPFNA exhibiting low-grade proliferative changes (Masood index, 11-12) which may reflect the overall breast cancer risk of this cohort. However, the presence of both M3 and M4 RARβ2 P2 promoter methylation in RPFNA was 15% for specimens with low-grade proliferative changes and 30% for RPFNA specimens exhibiting high-grade proliferative changes (Masood index, 13). Studies are in progress to test whether the presence of RARβ2 P2 methylation can be used to further risk stratify high-risk patients with proliferative changes.

The diverse sample of patients in this study and their variable clinical histories mirror the heterogeneous nature of the population of women at an increased risk of developing breast cancer. Such heterogeneity poses a challenge to clinicians in diagnosing early breast disease, risk-stratifying patients, and deciding on appropriate treatment. Therefore, identifying biomarkers that account for such diversity while providing information on possible disease progression is a

worthy goal. Our sample size is currently too small to provide further analysis comparing RARβ2 P2 promoter methylation to patients' clinical histories. We attempted to compare Gail model risk calculations to methylation status but found that due to how the Gail model is calculated we could not obtain risk estimates on a majority of our patients. Risk calculations could not be done on (a) patients with a history of DCIS/lobular carcinoma *in situ* or invasive breast cancer, as the model did not incorporate them; (b) patients who are known and suspected *BRCA1/2* mutation carriers, as their Gail values do not accurately reflect their risk (39); (c) patients below the age of 35, as they were also not included in the development of the model; and (d) African American patients, as there is concern that the Gail model does not accurately reflect risk in African American women (40). Thus, only 47% (18 of 38) of our subjects were eligible for the Gail model risk assessment, and their findings were not enough to achieve statistical significance. Further subset analysis could not be accurately done on methylation status relative to other factors, such as history of breast disease or proportion of atypical cells per sample, due to limited sample size. A small percentage of patients (5 of 38) were on prevention therapy at the time of aspiration (see Table 3B); two patients were not included in the analysis due to insufficient epithelial cell count, two patients only just started antiestrogen therapy 2 weeks before RPFNA, and the exclusion of the final patient's results did not statistically alter our findings. Our hope is to serially follow these patients via RPFNA and observe any cytologic changes that occur as a result of treatment.

These studies used MS-PCR to detect the presence of RARβ2 P2 promoter methylation at two target regions. MS-PCR is an established method for detecting methylation in DNA sequences. The method has been successfully used to examine methylation in relatively homogenous samples such as cancer

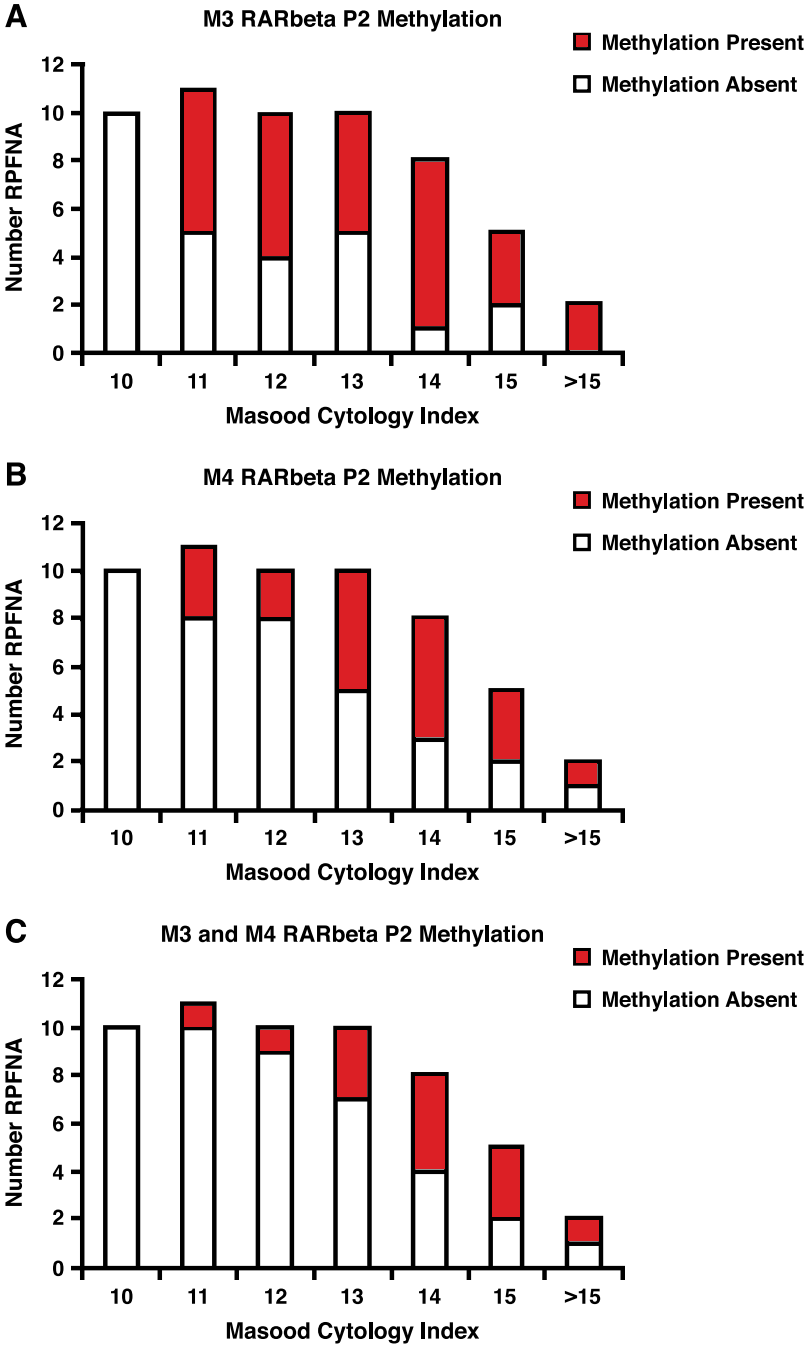

Figure 3. Correlation between RARβ2 P2 promoter methylation in RPFNA with Masood cytology index scores. RPFNA samples were assessed for cytologic atypia using the Masood cytology index. The distribution of RARβ2 P2 promoter methylation is depicted as a function of increased cytologic abnormality. **A.** Distribution of RFPNA samples with M3 region methylation relative to Masood cytology score. **B.** Distribution of RFPNA samples with M4 region methylation relative to Masood cytology score. **C.** RPFNA samples containing methylation at both the M3 and M4 regions relative to Masood cytology score.

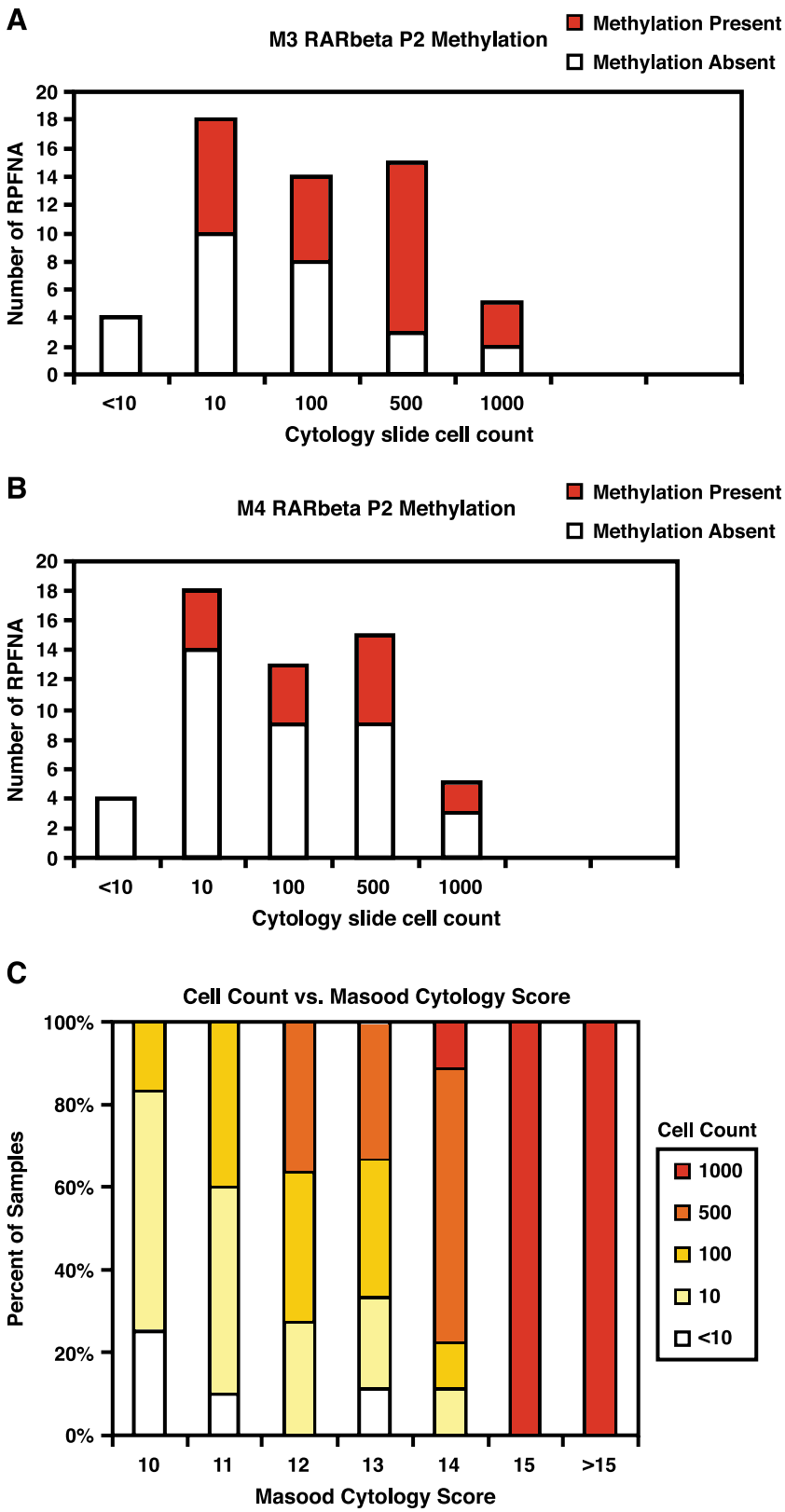

Figure 4. Correlation between RPFNA cell count and RARβ2 P2 promoter methylation and Masood score. **A** and **B.** Presence of RARβ2 P2 promoter methylation at the M3 (*A*) and M4 (*B*) region is depicted relative to total cell count of each RPFNA sample. **C.** RPFNA Masood cytology index scores are reported relative to the total cell count of each sample.

cell lines and tumor specimens. In such samples, primers anneal to plentiful ''target'' sequences readily. However, RPFNA samples consist of cells from the whole breast; a heterogeneous collection that may or may not include ''fields'' of increased risk. Assuming RARβ2 P2 promoter methylation is present in an RPFNA sample, MS-PCR primers would have far less ''target'' DNA to possibly bind, compared with in homogeneous samples with potentially greater amounts of target. MS-PCR in RPFNA samples is then complicated by two factors: (a) less target DNA and (b) a sea of presumably unmethylated DNA from the whole breast that partially impairs proper annealing of methylated primers. These factors are more easily overcome in cell lines and tumor specimens but require additional PCR optimization in RPFNA samples. It

is for these reasons that the PCR was optimized with such specific buffers and additives to refine the program and ensure small amounts of target-positive control could be amplified. Additionally, the cellular sensitivity experiment was set up for each region to gauge the effect of unmethylated DNA during amplification of the methylated target DNA. When optimized, the MS-PCR assay was able to detect 0.005% methylation at the M3 region and 0.05% methylation at the M4 region. Thus, the final optimized programs are quite sensitive, so each program was run in triplicate and only repeatable bands were counted as methylated.

Breast RPFNA has been used to predict both short-term breast cancer risk and monitor response to chemoprevention agents (25, 26). There are, however, some current limitations of breast RPFNA. First, much of the assessment of RPFNA samples has been focused on morphologic analysis. Molecular analysis of RPFNA samples may have the potential to enhance both the reproducibility and prognostic value of RPFNA. Second, many breast cancer prevention agents inhibit mammary cell proliferation and therefore have the potential to reduce the cell yield from RPFNA. As seen in our study as well as others' (29), MS-PCR studies on methylated genes such as *RARβ2* can still be done even when epithelial samples are inadequate for cytology. The statistically significant correlation between increasingly atypical morphologic appearance and the increase in rates of detectable methylation at the RARβ promoter site supports the concept that the methylation assay is accurately detecting an early step in carcinogenesis. The addition of MS-PCR–based marker analysis of RPFNA provides additional sensitivity to assist in determining whether a prevention agent (a) solely acted to decrease proliferation or (b) was also successful in eliminating abnormal cells. Thus, RPFNA coupled with methylation studies shows promise for the early detection of breast cancer risk and provides an extremely sensitive marker to track response to breast cancer prevention agents.

References

1. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
2. Rebbeck TR. Prophylactic oophorectomy in BRCA1 and BRCA2 mutation carriers. Eur J Cancer 2002;38 Suppl 6:S15–7.
3. Hartmann LC, Schaid DJ, Woods JE, et al. Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer. N Engl J Med 1999;340:77–84.
4. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer 2001;1:181–93.
5. Seewaldt VL, Johnson BS, Parker MB, et al. Expression of retinoic acid receptor β mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. Cell Growth Differ 1995;6:1077–88.
6. Subbaramaiah K, Cole PA, Dannenberg AJ. Retinoids and carnosol suppress cyclooxygenase-2 transcription by CREB-binding protein/p300-dependent and -independent mechanisms. Cancer Res 2002;62:2522–30.
7. Treuting PM, Chen LI, Buetow BS, et al. Retinoic acid receptor β2 inhibition of metastasis in mouse mammary gland xenografts. Breast Cancer Res Treat 2002;72:79–88.
8. Deng G, Lu Y, Zlotnikov G, et al. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 1996;274:2057–9.
9. Widschwendter M, Berger J, Daxenbichler G, et al. Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. Cancer Res 1997;57:4158–61.
10. Seewaldt VL, Kim JH, Caldwell LE, et al. All-*trans*-retinoic acid mediates G_1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ 1997;8:631–41.
11. Seewaldt VL, Caldwell LE, Johnson BS, et al. Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cellular proliferation and inhibits the formation of a polarized epithelium *in vitro*. Exp Cell Res 1997;236:16–28.
12. Dietze EC, Caldwell LE, Marcom K, et al. Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of CBP/p300. Microsc Res Tech 2002;59:23–40.
13. Kopelovich L, Crowell JA, Fay JR. The epigenome as a target for cancer chemoprevention. J Natl Cancer Inst 2003;95:1747–57.
14. Sirchia SM, Ferguson AT, Sironi E, et al. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor β2 promoter in breast cancer cells. Oncogene 2000;19:1556–63.
15. Widschwendter M, Berger J, Hermann M, et al. Methylation and silencing of the retinoic acid receptor-β2 gene in breast cancer. J Natl Cancer Inst 2000;92:826–32.
16. Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 2000;92:564–9.
17. Fackler MJ, McVeigh M, Evron E, et al. DNA methylation of RASSF1A, HIN-1, RAR-β, Cyclin D2 and Twist in *in situ* and invasive lobular breast carcinoma. Int J Cancer 2003;107:970–5.
18. Lapidus RG, Nass SJ, Butash KA, et al. Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Cancer Res 1998;58:2515–9.
19. Lehmann U, Langer F, Feist H, et al. Quantitative assessment of promoter hypermethylation during breast cancer development. Am J Pathol 2002;160:605–12.
20. Liu ZJ, Maekawa M, Horii T, et al. The multiple promoter methylation profile of PR gene and ERα gene in tumor cell lines. Life Sci 2003;73:1963–72.
21. Mancini DN, Rodenhiser DI, Ainsworth PJ, et al. CpG methylation within the 5′ regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. Oncogene 1998;16:1161–9.
22. Nass SJ, Herman JG, Gabrielson E, et al. Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. Cancer Res 2000;60:4346–8.
23. Sathyanarayana UG, Padar A, Suzuki M, et al. Aberrant promoter methylation of laminin-5-encoding genes in prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2003;9:6395–400.
24. Umbricht CB, Evron E, Gabrielson E, et al. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene 2001;20:3348–53.
25. Fabian CJ, Kimler BF, Zalles CM, et al. Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model. J Natl Cancer Inst 2000;92:1217–27.
26. Fabian CJ, Kimler BF. Beyond tamoxifen new endpoints for breast cancer chemoprevention, new drugs for breast cancer prevention. Ann N Y Acad Sci 2001;952:44–59.
27. Dooley WC, Ljung BM, Veronesi U, et al. Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. J Natl Cancer Inst 2001;93:1624–32.
28. Sirchia SM, Ren M, Pili R, et al. Endogenous reactivation of the RARβ2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455–61.
29. Evron E, Dooley WC, Umbricht CB, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001;357:1335–6.
30. Zalles CM, Kimler BF, Kamel S, et al. Cytology patterns in random aspirates from women at high and low risk for breast cancer. Breast J 1995;1:343–9.
31. Stampfer MR. Isolation and growth of human mammary epithelial cells. J Tissue Cult Meth 1985;9:107–15.
32. Seewaldt VL, Mrozek K, Dietze EC, et al. Human papillomavirus type 16 E6 inactivation of p53 in normal human mammary epithelial cells promotes tamoxifen-mediated apoptosis. Cancer Res 2001;61:616–24.
33. Seewaldt VL, Mrozek K, Sigle R, et al. Suppression of p53 function in normal human mammary epithelial cells increases sensitivity to extracellular matrix-induced apoptosis. J Cell Biol 2001;155:471–86.
34. Lehmann U, Kreipe H. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 2001;25:409–18.
35. Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res 2001;29:E65–5.
36. Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene 2002;21:5462–82.
37. Yang Q, Yoshimura G, Nakamura M, et al. Allelic loss of chromosome 3p24 correlates with tumor progression rather than with retinoic acid receptor β2 expression in breast carcinoma. Breast Cancer Res Treat 2001;70:39–45.
38. Youssef EM, Lotan D, Issa JP, et al. Hypermethylation of the retinoic acid receptor-β(2) gene in head and neck carcinogenesis. Clin Cancer Res 2004;10:1733–42.
39. Breast Cancer Risk Assessment Tool [homepage on the Internet]. Bethesda: National Cancer Institute; c2000 [cited 2004 Oct 13]. Available from: http://bcra.nci.nih.gov/brc/.
40. Bondy ML, Newman LA. Breast cancer risk assessment models: applicability to African-American women. Cancer 2003;97:230–5.

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