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Morphologically Normal-Appearing Mammary Epithelial Cells Obtained from High-Risk Women Exhibit Methylation Silencing of *INK4a/ARF*

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Abstract

Purpose: p16(INK4a) has been appreciated as a key regulator of cell cycle progression and senescence. Cultured human mammary epithelial cells that lack p16(INK4a) 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. These data suggest that cells that lack p16(INK4a) activity would be at high risk for breast cancer development and may exhibit an increased frequency of DNA methylation events in early cancer.

Experimental Design: To test this hypothesis, the frequencies of *INK4a/ARF* promoter hypermethylation, as well as four additional selected loci, were tested in the initial random periareolar fine needle aspiration samples from 86 asymptomatic women at high risk for development of breast cancer, stratified using the Masood cytology index.

Results: *INK4a/ARF* promoter hypermethylation was observed throughout all early stages of intraepithelial neoplasia and, importantly, in morphologically normal-appearing mammary epithelial cells; 29 of 86 subjects showed *INK4a/ARF* promoter hypermethylation in at least one breast. Importantly, *INK4a/ARF* promoter hypermethylation was not associated with atypia, and the frequency of hypermethylation did not increase with increasing Masood cytology score. The frequency of *INK4a/ARF* promoter hypermethylation was associated with the combined frequency of promoter hypermethylation of retinoic acid receptor-β2, estrogen receptor-α, and breast cancer-associated 1 genes ($P = 0.001$).

Conclusions: Because *INK4a/ARF* promoter hypermethylation does not increase with age but increases with the frequency of other methylation events, we predict that *INK4a/ARF* promoter hypermethylation may serve as a marker of global methylation dysregulation.

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p16(INK4a) acts to block cell cycle progression by binding to cyclin-dependent kinase-4 (CDK4) and CDK6 and inhibiting the catalytic activity of the CDK4-CDK6/cyclin D complex required for retinoblastoma protein phosphorylation (1, 2). p16(INK4a) blocks progression beyond the G_1-S restriction point by disrupting the formation of an E2F-DB active transcriptional complexes, thereby preventing the transcription of cell cycle progression genes (3). Loss or inactivation of p16(INK4a) function has been observed in numerous tumor types (4–6), and p16(INK4a) has been implicated to play an important role in the control of replicative senescence in fibroblasts and human mammary epithelial cells (7, 8).

Loss of p16(INK4a) function has been identified to be the result of both genetic and epigenetic events. Multiple mechanisms exist, including point mutation, loss of heterozygosity (LOH), small homozygous deletion (<200 kb), and promoter hypermethylation. LOH at the *INK4a/ARF* locus (9p21) has been reported in a number of neoplasias, including breast

Table 1. Masood cytology index scoring system

Cellular morphology	Cellular pleomorphism	Myoepithelial cells	Aniso-nucleosis	Nucleoli	Chromatin clumping	Score
Monolayer	Absent	Many	Absent	Absent	Absent	1
Nuclear overlap	Mild	Moderate	Mild	Occasional micronucleoli	Rare	2
Clustering	Moderate	Few	Moderate	Micronucleoli	Occasional	3
Loss cohesion	Conspicuous	Absent	Frequent	Macronucleoli	Frequent	4

NOTE: Sum of Masood scores: 6-10, normal; 11-13, proliferate (hyperplasia without atypia); 14-17, hyperplasia with atypia; >17, suspicious cytology.

(9, 10). Small homozygous deletion of *INK4a/ARF* is frequently observed in human breast cancer cell lines (29%, 4 of 14; refs. 5, 11), but reports are conflicting on its frequency in primary breast carcinomas (9, 10, 12). Promoter hypermethylation of *INK4a/ARF* has been observed in many cancer types. Hypermethylation of the promoter sequence is accompanied by suppression of gene expression, which is lifted after treatment with the demethylating agent 5-deoxyazacytidine (5). *INK4a/ARF* promoter hypermethylation has been observed in breast cancer specimens at rates varying from 4% to 55%, with the majority reporting a rate of 18% to 20% (9, 13–15).

Unlike many other loci reported to be hypermethylated in cancer, *INK4a/ARF* promoter hypermethylation has often been observed in focal patches of morphologically normal-appearing breast tissue obtained from reduction mammoplasty (15, 16). Human mammary epithelial cells lacking p16(INK4a) activity exhibit telomeric dysfunction (17), centrosomal dysfunction which generates aneuploidy, increased invasion and stimulation of angiogenesis, decreased apoptosis (18), and activation of a program for targeted DNA hypermethylation (19). Based on these observations, we and other investigators hypothesize that *INK4a/ARF* promoter hypermethylation might play an early role in breast cancer initiation.

If *INK4a/ARF* promoter hypermethylation is involved in cancer initiation and progression, one would expect its frequency to increase with increasing atypia. To test this hypothesis, the frequency of *INK4a/ARF* promoter hypermethylation was tested in random periareolar fine needle aspiration (RPFNA) samples obtained from 86 asymptomatic women at high risk for development of breast cancer and stratified using the Masood cytology index. 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 so as to assess both (*a*) breast cancer risk and (*b*) response to chemoprevention (20–22). RPFNA can be done successfully in a majority of high-risk women (82-89% cell yield; refs. 20–22). RPFNA samples were stratified using the Masood cytology index to indicate the level of cellular atypia. In this study, we show in high-risk women that *INK4a/ARF* promoter hypermethylation is observed in normal nonproliferating cells, epithelial hyperplasia, and hyperplasia with atypia; the latter two are often considered as the early stages of breast precancer or intraepithelial neoplasia (23). The frequency of *INK4a/ARF* promoter hypermethylation was associated with an increased combined frequency of promoter hypermethylation of retinoic acid receptor-β2 (*RARB*), estrogen receptor-α (*ESR1*), and breast cancer associated-1 (*BRCA1*) genes ($P = 0.001$). In this study, we did not observe an association between *INK4a/ARF* promoter hypermethylation and atypia in high-risk women. However, because *INK4a/ARF* promoter hypermethylation does not increase with age, but instead increases with the frequency of other methylation events, we predict that *INK4a/ARF* promoter hypermethylation may serve as a marker of methylation dysregulation in high-risk women.

The phenomenon of a CpG island methylator phenotype has been well described in colorectal cancer (24–26). However, there is little evidence for CpG island methylator phenotype in breast cancer (27, 28). By examining a panel of methylation markers, we tested for CpG island methylator phenotype in early mammary carcinogenesis. This study provides a potential link between *INK4a/ARF* promoter hypermethylation and CpG island methylator phenotype in breast cancer.

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.

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 of ≥1.7%, (*b*) prior biopsy exhibiting atypical hyperplasia, lobular carcinoma *in situ*, or ductal carcinoma *in situ*, (*c*) known BRCA1/BRCA2 mutation carrier, or (*d*) contralateral breast cancer (25).

Mathematical assessment of breast cancer risk. Gail model and BRCAPRO score assessments were done using the Breast Cancer Risk Assessment Tool[6] and *CancerGene*[7] 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-matched and race-matched peers (31). Women under age of 35 years are not appropriate for Gail risk calculation. We did not perform Gail risk calculation for African-American women because we were concerned about 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, including second-degree relatives, of breast and/or ovarian cancers (32).

RPFNA. RPFNA was done as previously published (20–22). 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 (20, 21). Cells were classified

[6] http://bcra.nci.nih.gov/

[7] http://www3.utsouthwestern.edu/cancergene/

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 (20, 34; see Table 1). The number of epithelial cells was quantified and classified as <10 cells (insufficient quantity for cytologic analysis), 10 to 100 cells, 100 to 500 cells, 500 to 1,000 cells, 1,000 to 5,000 cells, and >5,000 cells. Morphologic assessment, Masood cytology index scores, and cell count were assigned by a blinded, single, dedicated pathologist (C.M.Z.; refs. 20–22).

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. The MDA-MB-453 breast cancer cell line was obtained from the laboratory of Sally Kornbluth and grown in supplemented αMEM (Life Technologies; ref. 35).

DNA extraction and bisulfite treatment. DNA was extracted from breast cancer cell lines and RPFNA as previously published; bisulfite treatment was as previously published (22).

LOH. We tested for LOH at the *INK4a/ARF locus* 9p21 in 23 of 41 atypical (Masood score, 15-17) RPFNA specimens. Testing was not done in the remaining 18 atypical specimens due to either the subject's refusal of blood draw or an inability to cannulate the subject's vein on three successive attempts as per protocol. In addition, LOH analysis was done on 10 hyperplastic samples (Masood score, 11-13). Three microsatellite markers that map to 9p21 were used (see Fig. 2D): D9S916, D9S974, and D9S942. Primer sequences are as previously published (36). All PCR reactions consisted of 50 ng genomic DNA, 13 PCR buffer (Qiagen), 250 μmol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 2.5 units of HotStar Taq polymerase (Qiagen) in 30 μL of total volume. Patient RPFNA sample and matched blood sample were tested in triplicate for each microsatellite locus. For each microsatellite locus, the marker was determined to be uninformative, informative with LOH, or informative without LOH. If the marker locus was informative (heterogeneous), the height of each peak representing an allele was obtained. The ratios of the two alleles from the whole blood and RPFNA DNA samples were determined, and the average of these ratios from the triplicate PCR was obtained. The ratio of the two alleles was determined by densitometry. The ratio for chromosomal loss was chosen at 0.70 (loss of 30% or greater; refs. 36, 37).

Methylation-specific PCR. Previous work has elucidated appropriate methylation-specific PCR (MSP) primers within exon 1α of the *INK4a/ARF* promoter (38–40). Primer sequences are as previously published (38). All PCR reactions consisted of 50 ng bisulfite-treated DNA, 1× PCR buffer, 250 μmol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 2.5 units of HotStar Taq polymerase (Qiagen) in 30 μL total volume. 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 MDA-MB-453 genomic DNA for a total of 1 μg.

Four other MSP promoter hypermethylation targets were tested, including *RARB* at M3 (nt -51 to nt +162) and M4 (nt +104 to nt +251; ref. 27), *BRCA1* (nt -150 to nt +32; ref. 41), and *ESR1* (nt +367 to nt +494; ref. 42). MSP conditions and primers for *RARB* (M3 and M4) were as previously published (22). MSP primers for *BRCA1* were as published (41), except an annealing temperature of 63°C was used in both the M and U programs. MSP primers for *ESR1* were as published (primer pair 5; ref. 42), except annealing temperatures of 56°C and 52°C were used in the M and U programs. The full cohort was not tested in this analysis due to insufficient sample.

Statistical methods. The Wilcoxon rank-sums test was used to compare median age, Gail score, BRCAPRO score, Masood cytology index score, RPFNA cell count, family history of cancer, and hypermethylation of four additional sites in the promoters of the *RARB*, *ESR1*, and *BRCA1* genes with *INK4a/ARF* promoter hypermethylation. The Spearman correlation coefficient was used to determine the association between cell count and Masood cytology index score. *INK4a/ARF* promoter hypermethylation was also compared with age.

Results

Study demographics. One hundred six women underwent initial RPFNA at Duke University Medical Center from March 2003 to August 2005 (Table 2). Of the 162 RPFNA samples that were collected, 42 samples had insufficient epithelial cells for cytologic testing. Twenty of the original 106 women lacked a minimum of one RPFNA sample with a sufficient number of epithelial cells for analysis. Therefore, 120 RPFNA samples (162 total samples less 42 insufficient samples) from 86 subjects (106 subjects less 20 subjects lacking a minimum of one RPFNA sample with sufficient epithelial cells) were submitted for full cytologic analysis. Fifty percent (43 of 86) of subjects had bilateral RPFNA. Because RPFNA was not done on radiated breast tissue, 50% (43 of 86) had unilateral RPFNA. Eighty-six percent (74 of 86) of the women were Caucasian and 14% (12 of 86) were African-American.

Table 2. Patient characteristics for RPFNA

Women enrolled in study	106
Bilateral RPFNA	56
Unilateral RPFNA	50
RPFNA samples collected	162
RPFNA samples with insufficient epithelial cell count	42
Subjects lacking a minimum of one RPFNA sample with sufficient epithelial cell count	20
RPFNA samples (from subjects) submitted for analysis	120 (86)
	***n* = 86**
Average age and range (y)	47 (29-66)
Race	
Caucasian	74 (86%)
African-American	12 (14%)
Menopausal status	
Postmenopausal	26 (30%)
Premenopausal/perimenopausal	60 (70%)
Hormone replacement use	
Current	2 (2%)
Ever-use	12 (14%)
Never-use	72 (84%)
Antiestrogen therapy (at the time of RPFNA)	
Tamoxifen	2 (2%)
Raloxifene	2 (2%)
Aromatase inhibitor	2 (2%)
Family history of breast cancer	52 (60%)
Prior abnormal biopsies	
ADH	13 (15%)
LCIS	4 (5%)
DCIS	11 (13%)
Hx. contralateral breast cancer	16 (19%)
Known BRCA1 mutation carriers	2
Known BRCA2 mutation carriers	2

Abbreviations: LCIS, lobular carcinoma *in situ*; DCIS, ductal carcinoma *in situ*.

Distribution of Masood cytology index and cell count. One hundred twenty RPFNA specimens from 86 women were stratified using the Masood cytology index (Fig. 1A). For the purpose of this analysis, RPFNA samples obtained from different breasts in the same individual were counted as separate samples because Masood scores for each breast were not concordant for all women. The median Masood cytology index score of RPFNA specimens was 13 (range, 8-19). The distribution of RPFNA epithelial cell count was determined and, as previously observed, positively correlated with RPFNA Masood cytology index score ($P < 0.001$; Fig. 1B; ref. 27).

INK4a/ARF promoter hypermethylation in morphologically normal-appearing mammary epithelial cells. Hypermethylation from nt +167 to nt +317 of the *INK4a/ARF* promoter was tested using MSP in 120 RPFNA specimens obtained from 86 high-risk women; for the purpose of this analysis, RPFNA samples obtained from different breasts in the same individual were counted as separate samples (Fig. 2A). This region includes exon 1α (38) and uses cytosines whose methylation indicates transcriptional repression. 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; Fig. 2B). At this level of sensitivity, MSP analysis showed *INK4a/ARF* promoter hypermethylation in 28% (36 of 120) of RPFNA samples. Of the 43 of 86 subjects who underwent bilateral RPFNA, 16% (7 of 43) exhibited bilateral INK4a/ARF promoter hypermethylation, 28% (12 of 43) exhibited unilateral *INK4a/ARF* promoter hypermethylation, and 56% (24 of 43) did not exhibit *INK4a/ARF* promoter hypermethylation in either breast. Strong unmethylated bands were detected in all included samples, confirming both the presence of DNA and the promoter sequence itself (Fig. 2C).

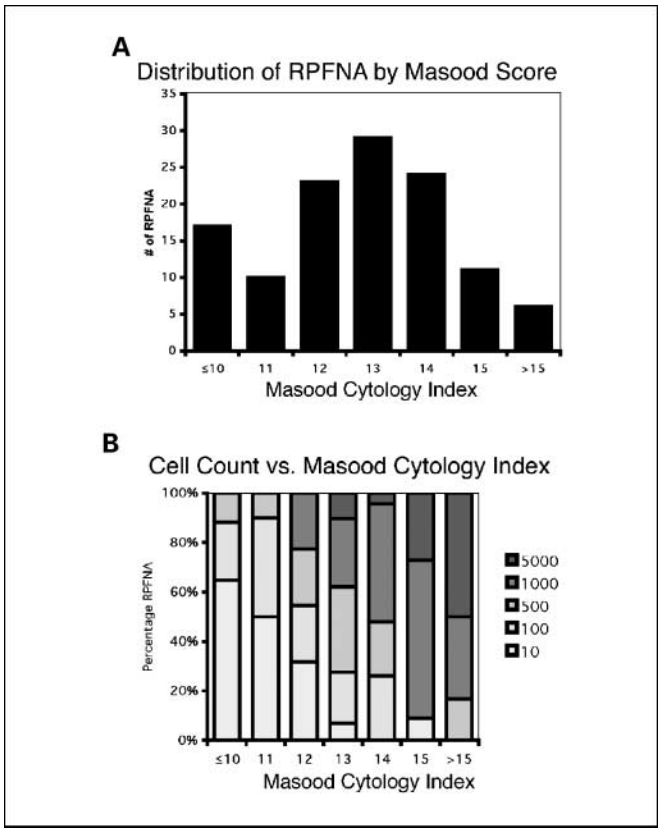

Fig. 1. Cytologic abnormality in RPFNA. RPFNA specimens were assessed for cell count and Masood cytology index score. *A,* the distribution of Masood cytology index scores for RPFNA specimens from high-risk women is depicted. *B,* Masood cytology index is also reported relative to the total cell count category of each RPFNA specimen.

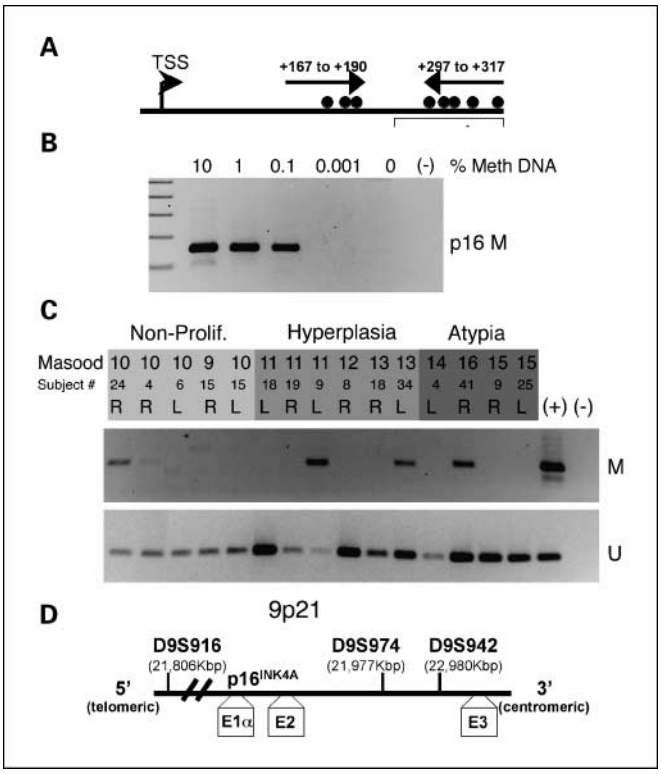

Fig. 2. *INK4a/ARF* promoter hypermethylation in RPFNA. *A,* MSP primers were designed to amplify the known potential hypermethylation region of the *INK4a/ARF* promoter (38). The region (nt +167 to nt +317) includes exon 1α (38) and uses cytosines whose methylation indicates transcriptional repression (26). Methylated CpGs to which MSP primers bind (*filled circles*). *B,* 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). Titration experiments were as described in Materials and Methods; p16 M, hypermethylation of the *INK4a/ARF* promoter. *C,* hypermethylation of the *INK4a/ARF* promoter in RPFNA obtained from 15 representative high-risk women with nonproliferative, hyperplastic, or atypical RPFNA. #, subject's identification number; M and U, the use of MSP primers to identify methylated and unmethylated *INK4a/ARF* promoter, respectively; (+), hypermethylated positive control in the M gels and the MDA-MB-453 breast cancer cell line in the U gels; (-), negative control. *D,* LOH was tested in atypical RPFNA specimens. The microsatellite markers D9S916, D9S974, and D9S942 were used; D9S916 is 5′ (telomeric) to the *INK4a/ARF* locus, whereas D9S974 and D9S942 lie within the coding sequence of the gene (36). E, exon.

INK4a/ARF promoter hypermethylation versus Masood cytology index. RPFNA samples were stratified using the Masood cytology index. One hundred twenty RPFNA samples were tested from 86 women. For the purpose of this analysis, RPFNA samples obtained from different breasts in the same individual were counted as separate samples. The distribution of *INK4a/ARF* promoter hypermethylation was reported as a function of increased cytologic abnormality (Fig. 3A). *INK4a/ARF* promoter hypermethylation was observed in 24% (4 of 17) of nonproliferative (normal; Masood score, ≤ 10), 26% (16 of 62) of hyperplastic (Masood score, 11-13), and 39% (16 of 41) of atypical cytology. Of the 7 of 43 cases that exhibited bilateral *INK4a/ARF* promoter hypermethylation, six of the seven cases contained at least one RPFNA sample that showed atypical cytology (Masood score, 14-17). The Masood score did not

differ between *INK4a/ARF* methylated and unmethylated samples (P = 0.24); both groups had a median Masood score of 13. *INK4a/ARF* promoter hypermethylation was compared with RPFNA cell count (Fig. 3B). The group of 93 unmethylated and 36 methylated samples had a median cell count category of 100 versus 500 cells, respectively; these two groups were significantly different from each other (P = 0.038). These combined observations show that the incidence of *INK4a/ARF* promoter hypermethylation is observed throughout early intraepithelial neoplasia, and the presence of *INK4a/ARF* promoter hypermethylation does not predict increased Masood cytology index score or the presence of cytologic atypia.

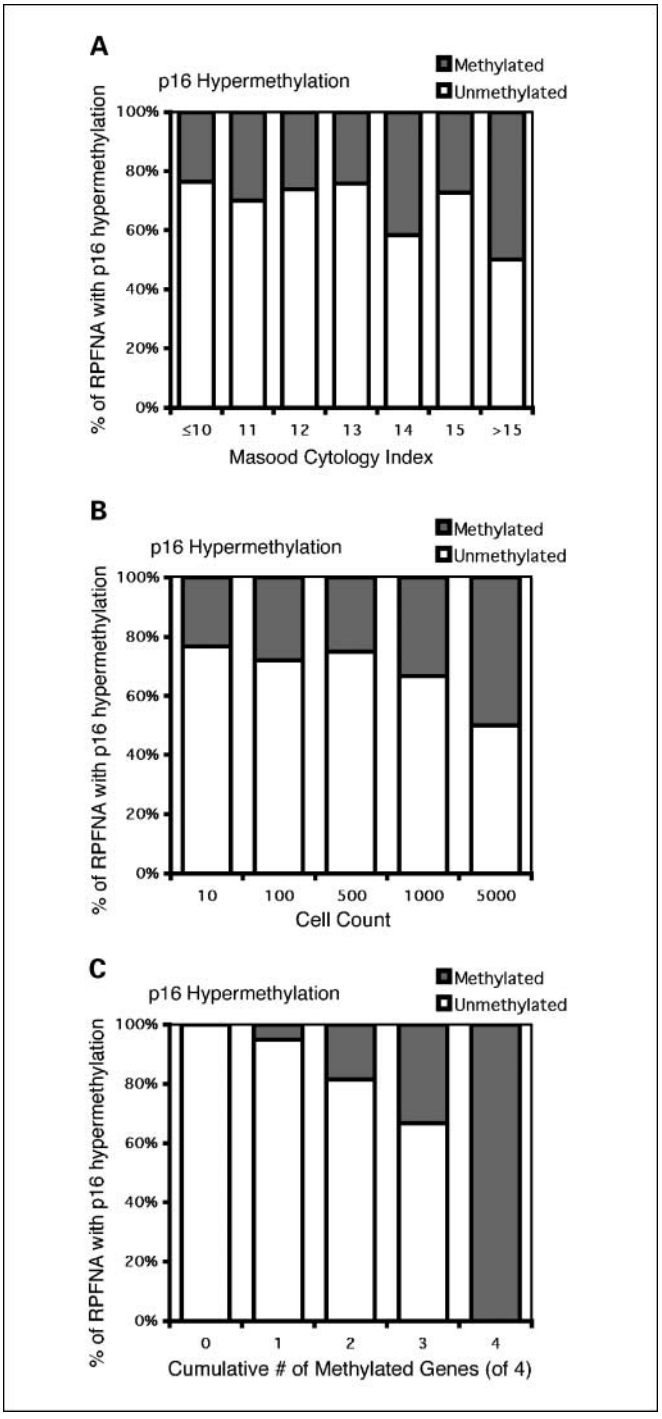

Fig. 3. *INK4a/ARF* promoter hypermethylation and cytology. RPFNA specimens obtained from high-risk women were tested for *INK4a/ARF* promoter hypermethylation. *A and B,* the distribution of RPFNA specimens with *INK4a/ARF* promoter hypermethylation is depicted relative to Masood cytology index score (*A*) and cell count category (*B*). *C,* the distribution of RPFNA specimens with p16(INK4a) promoter hypermethylation is depicted relative to the methylation status of four other sites using MSP, including *RARB* (at M3 and M4; ref. 27), *BRCA1* (41), and *ESR1* (42).

LOH of INK4a/ARF is not frequently observed in atypical RPRNA. LOH is also known to be a mechanism for loss of *INK4a/ARF* function. We tested for LOH at the *INK4a/ARF* locus 9p21 in 10 hyperplastic and 23 atypical RPFNA specimens obtained from 31 individual women. For the purpose of this analysis, RPFNA samples obtained from different breasts in the same individual were counted as separate samples. Three microsatellite markers were used: D9S916, D9S974, and D9S942. D9S916 is telomeric (5′) to *INK4a/ARF*, whereas D9S942 and D9S974 lie within the coding sequence of the gene (Fig. 2D). Markers D9S916, D9S974, and D9S942 were found informative in 20 of 33 (61%), 25 of 33 (76%), and 18 of 33 (55%) samples, respectively. The average ratios at all three microsatellite loci were compared with the cutoff value of 0.70 (loss of 30% or greater) to define LOH (36, 37). LOH of *INK4a/ARF* was present in only one microsatellite marker for 1 of 23 (4.3%) atypical RPFNA samples: 0 at D9S916, 1 at D9S974, and 0 at D9S942. In 10 hyperplastic RPFNA specimens tested, LOH of *INK4a/ARF* was not present in any microsatellite marker. These results show that LOH of *INK4a/ARF* is not frequently observed in atypical RPFNA cytology.

Frequency of INK4a/ARF promoter hypermethylation in RPFNA does not increase with age. The presence of *INK4a/ARF* promoter hypermethylation was tested as a function of family history of cancer, Gail model risk score, and age. The association was tested for individual women and not for individual RPFNA samples. To perform this analysis, subjects were considered hypermethylated for *INK4a/ARF* if promoter hypermethylation has detected RPFNA cytologic samples from either (*a*) one breast (unilateral hypermethylation) or (*b*) both breast (bilateral hypermethylation). No associations were found between *INK4a/ARF* promoter hypermethylation and family history of breast cancer (P = 0.78), premenopausal breast cancer (P = 0.91), or ovarian cancer (P = 0.23). Due to the limitations of the Gail model, only 57% (49/86) of subjects could be assessed. We did not calculate a Gail Model score in 43% of individuals because of a prior history of contralateral breast cancer, ductal carcinoma *in situ*, the subject was <35 years of age, or due to the Gail model underestimating risk in African-American subjects. There was no significant correlation between the frequency of *INK4a/ARF* promoter hypermethylation and the 5-year Gail model risk score (P = 0.94). No association was found between *INK4a/ARF* promoter hypermethylation and age (P = 0.78; Fig. 4).

INK4a/ARF promoter hypermethylation in RPFNA is inversely associated with BRCAPRO model score. The association between *INK4a/ARF* promoter hypermethylation and BRCAPRO model score was tested. To perform this analysis, subjects were considered hypermethylated for *INK4a/ARF* if promoter hypermethylation was detected in either (unilateral) or both (bilateral) RPFNA cytologic samples. The frequency of *INK4a/ARF* promoter hypermethylation was inversely associated with the likelihood of an individual carrying a *BRCA1* (P = 0.061) or *BRCA2* (P = 0.034) mutation as measured by BRCAPRO. There

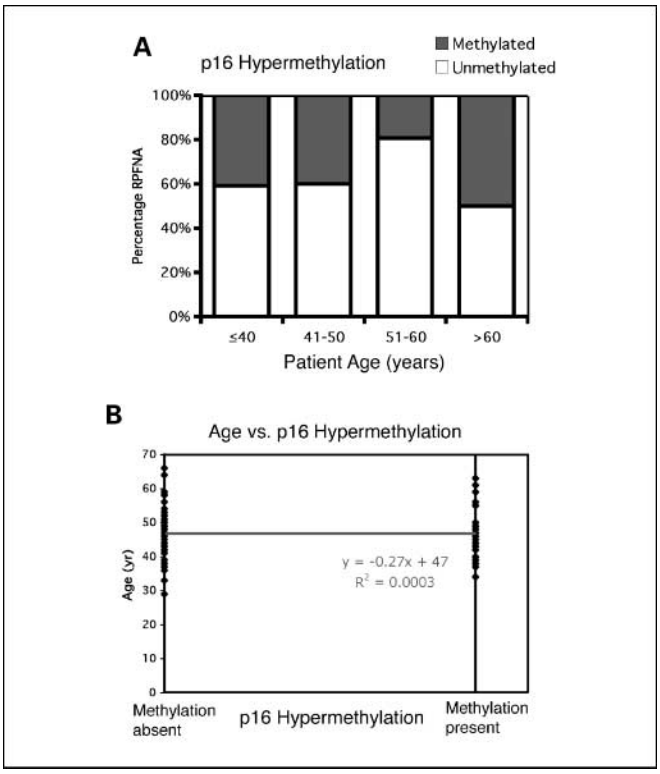

Fig. 4. *INK4a/ARF* promoter hypermethylation and age. RPFNA specimens obtained from high-risk women were tested for *INK4a/ARF* promoter hypermethylation as a function of age. The distribution of RPFNA specimens with *INK4a/ARF* promoter hypermethylation is depicted relative to age groupings.

was a significant difference in BRCAPRO scores between subjects with unmethylated *INK4a/ARF* (median BRCAPRO1 and BRCAPRO2 scores of 0.001 and 0.0008, respectively) and subjects with methylated *INK4a/ARF* (median BRCAPRO1 and BRCAPRO2 scores of 0.0005 and 0.0003, respectively). The mechanism for this inverse association is unclear, and further studies are required.

INK4a/ARF promoter hypermethylation predicts increased frequency of additional methylation events. The association between *INK4a/ARF* promoter hypermethylation and hypermethylation of four additional MSP markers was tested in 71 RPFNA samples from 54 subjects. The four other MSP promoter hypermethylation targets were *RARB* at M3 and M4 (27), *BRCA1* (41), and *ESR1* (42). These markers were evaluated in addition to *INK4a/ARF*. *INK4a/ARF* promoter hypermethylation was associated with a high frequency of methylation of other markers (P = 0.002; Fig. 3C). The distribution of all markers is shown as a function of Masood cytology (71 RPFNA samples) and age (54 subjects; Tables 3 and 4). In 100% of the samples (10 of 10 samples), wherein *INK4a/ARF* promoter hypermethylation was detected, at least one of the other four loci was also hypermethylated. In 2 of 10 cases, one of the other four loci were hypermethylated; in 5 of 10 cases, two of the other four loci were hypermethylated; in 2 of 10 cases, three of the other loci four loci were hypermethylated; and in one case, 1 of 10, all four loci were hypermethylated in addition to *INK4a/ARF*. In contrast, in the 61 samples that did not contain *INK4a/ARF* promoter hypermethylation, 0 of 61 samples were methylated at all four other loci, 3 of 61 samples were methylated at three loci, 16 of 61 samples were methylated at two loci, 28 of 61 samples were methylated at one locus, and 14 of 61 samples exhibited no methylation at any of the other four loci. These observations show that *INK4a/ARF* promoter hypermethylation is tightly associated with an increased frequency of *RARB*, *ESR1*, and *BRCA1* promoter hypermethylation.

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 hypermethylation in tumor suppressor gene silencing during early mammary carcinogenesis. Although evidence suggests that *INK4a/ARF* promoter hypermethylation is an important early event in mammary carcinogenesis, the frequency and distribution of *INK4a/ARF* promoter hypermethylation, as well as hypermethylation at other loci, in mammary epithelial cells from high-risk women is not known.

Here, we observe that *INK4a/ARF* promoter hypermethylation occurs frequently in mammary epithelial cells obtained from high-risk women. *INK4a/ARF* promoter hypermethylation is observed in specimens of all Masood scores, including nonproliferative (normal) and hyperplastic mammary epithelial cells. We do not see an association between *INK4a/ARF* promoter hypermethylation and increased Masood cytology index. In contrast with many methylation markers, for

Table 3. Multimarker methylation analysis in 71 RPFNA samples from 54 subjects: distribution of methylation markers versus Masood cytology index

Markers tested	Percentage of hypermethylated samples for Masood score range (n)				
	≤10 (n = 12)	11-12 (n = 21)	13 (n = 21)	14 (n = 8)	≥15 (n = 9)
p16	25% (3)	8% (2)	8% (2)	13% (1)	22% (2)
RARB M3	33% (4)	62% (13)	71% (15)	63% (5)	66% (6)
RARB M4	8% (1)	19% (4)	24% (5)	25% (2)	56% (5)
ESR1	33% (4)	14% (3)	15% (3)	13% (1)	44% (4)
BRCA1	25% (4)	24% (5)	0% (0)	13% (1)	22% (2)

NOTE: A total of 71 samples were tested for each methylation marker; n denotes the number of samples tested for each Masood cytology score range. For example, in Masood score of ≤10 (n = 12), 12 samples with a Masood cytology score were tested; of these 12 samples, three samples contained hypermethylation of the p16 promoter.

Table 4. Multimarker methylation analysis in 71 RPFNA samples from 54 subjects: distribution of methylation markers versus age

	Percentage of hypermethylated samples for age range (*n*)		
	≤40 y (*n* = 12)	41-50 y (*n* = 25)	51-64 y (*n* = 17)
p16	33% (4)	12% (3)	18% (3)
RARB M3	83% (10)	68% (17)	71% (15)
RARB M4	25% (3)	24% (6)	35% (6)
ESR1	0% (0)	7% (7)	41% (7)
BRCA1	6% (1)	20% (5)	24% (4)

NOTE: A total of 54 samples were tested for each methylation marker; *n* denotes the number of samples tested for each age range. For example, for ages ≤40 y, 12 samples were tested; of these 12 samples, four samples contained hypermethylation of the p16 promoter.

example *BRCA1* (43), the frequency of *INK4a/ARF* promoter hypermethylation does not increase with increasing age. LOH, which is found relatively frequent in invasive breast cancer (44, 45), is rare in RPFNA specimens and supports the idea that promoter hypermethylation occurs before genomic rearrangements.

Here, we tested in RPFNA samples obtained from high-risk women in our cohort for the association between *INK4a/ARF* promoter hypermethylation and hypermethylation of three genes that play key roles in breast cancer, including (*a*) *RARB* (RARβ2), (*b*) *ESR1* (ERα), and (*c*) *BRCA1*. We tested for *RARB* promoter hypermethylation because RARβ2 is a key regulator of proliferation and apoptosis and is a tumor suppressor in breast cancer (35, 46, 47). Whereas LOH is a late event, hypermethylation of the RARβ2 P2 promoter at the M3 and M4 sites is observed during early mammary carcinogenesis, and the frequency of RARβ2 P2 promoter hypermethylation increases strikingly with the frequency of cytologic atypia (22). Likewise, estrogen signaling plays an important role in mammary carcinogenesis, and ERα exhibits cross-talk with RARβ2, as well as other steroid thyroid receptors. The *ESR1* (ERα) promoter and first exon contain a CpG island, in which aberrant hypermethylation occurs in breast, endometrial, prostate, and lung cancer. In breast cancer cell lines, *ESR1* promoter hypermethylation exhibits a tight inverse relationship with ERα expression (42), and there is evidence that *ESR1* promoter hypermethylation predicts clinical response to tamoxifen. Finally, we also chose to examine the *BRCA1* promoter because hypermethylation of this important tumor suppressor locus is hypothesized to be a second mechanism for BRCA1 inactivation (44).

In this study, we did not observe an association between *INK4a/ARF* promoter hypermethylation and atypia in high-risk women. We observed that *INK4a/ARF* promoter hypermethylation in RPFNA cytology is associated with an increased frequency of *RARB*, *ESR1*, and *BRCA1* promoter hypermethylation ($P = 0.001$). Because *INK4a/ARF* promoter hypermethylation increases with the frequency of other methylation events but not age, we hypothesize that *INK4a/ARF* promoter hypermethylation may serve as a marker of global methylation dysregulation. The role of *INK4a/ARF* in methylation dysregulation in low-risk women cannot be determined by this study. The combination of *INK4a/ARF* promoter hypermethylation and increased promoter hypermethylation of *RARB*, *ESR1*, and *BRCA1* in high-risk women would be predicted to set the stage for further tumor progression.

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