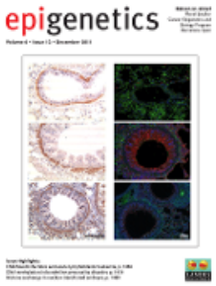

Epigenetics

ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: https://www.tandfonline.com/loi/kepi20

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To cite this article: Eva P. Browne, Elizabeth C. Punska, Sarah Lenington, Christopher N. Otis, Douglas L. Anderton & Kathleen F. Arcaro (2011) Increased promoter methylation in exfoliated breast epithelial cells in women with a previous breast biopsy, Epigenetics, 6:12, 1425-1435, DOI: 10.4161/epi.6.12.18280

To link to this article: https://doi.org/10.4161/epi.6.12.18280

RESEARCH PAPER

Epigenetics 6:12, 1425-1435; December 2011; © 2011 Landes Bioscience

Increased promoter methylation in exfoliated breast epithelial cells in women with a previous breast biopsy

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Key words: promoter methylation, pyrosequencing, breast milk, biopsy, GSTP1, RASSF1, SFRP1, breast epithelial cells, breast cancer risk

Abbreviations: CpG, cytosine phosphodiester bonded to guanine; GSTP1, glutathione-*S*-transferase pi; MACS, magnetic antibody cell separation; RASSF1, rass association domain family 1; SFRP1, secreted frizzle related protein 1

Accurately identifying women at increased risk of developing breast cancer will provide greater opportunity for early detection and prevention. DNA promoter methylation is a promising biomarker for assessing breast cancer risk. Breast milk contains large numbers of exfoliated epithelial cells that are ideal for methylation analyses. Exfoliated epithelial cells were isolated from the milk obtained from each breast of 134 women with a history of a non-proliferative benign breast biopsy (Biopsy Group). Promoter methylation of three tumor suppressor genes, *RASSF1*, *SFRP1* and *GSTP1*, was assessed by pyrosequencing of bisulfite-modified DNA. Methylation scores from the milk of the 134 women in the Biopsy Group were compared to scores from 102 women for whom a breast biopsy was not a recruitment requirement (Reference Group). Mean methylation scores for *RASSF1* and *GSTP1* were significantly higher in the Biopsy than in the Reference Group. For all three genes the percentage of outlier scores was greater in the Biopsy than in the Reference Group but reached statistical significance only for *GSTP1*. A comparison between the biopsied and non-biopsied breasts of the Biopsy Group revealed higher mean methylation and a greater number of outlier scores in the biopsied breast for both *SFRP1* and *RASSF1*, but not for *GSTP1*. This is the first evidence of CpG island methylation in tumor suppressor genes of women who may be at increased risk of developing breast cancer based on having had a prior breast biopsy.

Introduction

It is estimated 230,480 women will be diagnosed with breast cancer and 39,520 will die from the disease in the US in 2011.[1] Despite significant advances in early detection and improved treatment, breast cancer remains the second most diagnosed cancer and the second leading cause of cancer deaths in women.[1] Recent guidelines by the US Preventive Services Task Force recommend screening mammography begin at age 50 rather than the previously recommended age 40 for all women at average risk and that "the decision to start regular, biennial screening mammography before the age of 50 years should be an individual one and take patient context into account, including the patient's values regarding specific benefits and harms."[2] This recommendation assumes the individual has an understanding of her risk of developing breast cancer.

Epidemiological studies have consistently identified established risk factors, including early menarche, nulliparity, late age at first birth, previous biopsy and family history as contributing to breast cancer incidence. However, these same studies find that almost all women have at least one of the established risk factors.[3-6] Furthermore, while women with a family history of breast cancer are at increased risk, most women with a family history of breast cancer do not develop the disease. A collaborative reanalysis of 52 epidemiological studies found that 8 out of 9 women who develop breast cancer do not have an affected mother, sister or daughter.[7] The NCI estimates that only 5–15% of all breast cancers are associated with mutations in autosomal dominant genes such as BRCA1, BRCA2, Tp53 and PTEN.[1,7] The vast majority of breast cancers are sporadic, making it extremely difficult for women to assess their individual breast cancer risk.

There is a great desire and need to find molecular markers that accurately predict individual breast cancer risk. DNA methylation of tumor suppressor genes is considered one of the most promising of the molecular biomarker candidates.[8,9] Methylation of CpG islands in promoter regions frequently results in transcriptional silencing of the gene and is thought to occur early in the etiology of breast cancer.[10] Most importantly, CpG island

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Submitted: 08/18/11; Revised: 09/29/11; Accepted: 10/02/11
DOI: 10.4161/epi.6.12.18280

methylation is potentially reversible with drug treatment or diet modification.[11] However, the promoter methylation pattern characteristic of cancer, early stage disease or increased risk is tissue specific. That is to say, methylation associated with breast cancer risk must be assessed in breast tissue.

Studies examining the relationship between promoter methylation and breast cancer risk have used DNA from cells in nipple aspirate, ductal lavage and random periareolar fine-needle aspirate (PFNA).[12-15] While these studies have provided important information, there are several drawbacks to these sampling methods such as, the amount of material obtained being small (e.g., nipple aspirate and ductal lavage), the material obtained being from a limited area or single lobule of the breast (all sampling methods) and method of collection being invasive and not likely to be widely accepted (PFNA and ductal lavage).[14,15] In contrast, breast milk contains millions of epithelial cells representing all of the tissue in the entire mammary gland and can be easily obtained from each breast as exfoliated epithelial cells are naturally present in breast milk. Women are likely to participate in studies of breast milk because collecting milk is non-invasive. Additionally, women who have multiple pregnancies can contribute samples over a number of years, providing important information regarding methylation changes over time.

Promoter methylation of the three genes analyzed in the present study has been detected previously in breast cancer, and may increase a woman's risk of developing this disease. *RASSF1* (*Rass association domain family1 protein*), a tumor suppressor gene implicated in the development of breast cancer,[16] promotes apoptosis and cell cycle arrest, and reduces the tumorigenicity of cancer cell lines.[17] Aberrant promoter methylation of *RASSF1* is frequently detected in breast cancer tissue[18-21] and has also been detected in serum of breast cancer patients[22] and in fine needle aspirate from benign epithelium of women at high risk for breast cancer.[23] *SFRP1* (*Secreted frizzle related protein 1*) is a tumor suppressor gene encoding a WNT signaling antagonist abundantly expressed in normal breast tissue. *SFRP1* has been reported to be hypermethylated in >70% of breast cancers.[24] *GSTP1* (Glutathione-*S*-transferase *P1*) plays a role in protecting cells from cytotoxic and carcinogenic agents.[25] Expression of GSTP1 varies from tissue to tissue; however, loss of *GSTP1* expression has been attributed to DNA hypermethylation.[26] Methylation of *GSTP1* has been detected in breast cancer tissue[18,27-30] as well as breast fluids from cancer patients[13,31] and has been correlated with age in benign prostate.[32]

We have chosen to study methylation changes in candidate biomarker genes (*RASSF1*, *SFRP1* and *GSTP1*) and their relationship to breast cancer risk using milk from nursing women. Since most women do not develop breast cancer, the vast majority of nursing mothers are not expected to have increased CpG island promoter methylation. Accordingly, we previously demonstrated that mean methylation levels were extremely low in the exfoliated epithelial cells isolated from breast milk of women at no known increased risk of developing breast cancer.[33] To determine whether methylation analysis of cells in breast milk can be used to assess breast cancer risk it is important to select a population in which it is probable that a subset of participants will be at a significantly increased risk for developing breast cancer. Our approach is to recruit nursing mothers who either have had a breast biopsy or are scheduled to have a breast biopsy due to a suspicious lump. This is an ongoing study in which we continue to recruit women and acquire annual follow-up information. In the present report, we compare the methylation scores obtained from cells in the milk of 134 women whose breast biopsy indicated the lowest risk, benign, non-proliferative lesion, and compare these methylation scores with those from a reference group who were not selected based on biopsy history. Among women who had a breast biopsy we also compare the methylation scores obtained from their biopsied and non-biopsied breasts. Results show increased methylation in the Biopsy Group and considerable individual variability. Only long-term follow-up will reveal if these individual methylation scores are accurate predictors of breast cancer risk.

Results

Subject recruitment. In the Biopsy Group, milk samples were collected and processed from 141 consented women whose biopsy reports indicated they had a non-proliferative lesion (Category 1). The majority of biopsies were surgical and core (47 and 38%, respectively) followed by FNA (10%), and by far the most frequent diagnosis was fibroadenoma (40%) followed by lactational changes, fibrocystic change and fibrosis (**Table 1**). Of the ten women who received a "negative" diagnosis, five of these diagnoses were based on a FNA biopsy and were considered insufficient to retain the women in the low risk Category 1. Of the remaining 135 women in Category 1, epithelial-enriched cell fractions were obtained from the milk of at least one breast from 134 women. For the Reference Group, milk samples were obtained from 111 consented women and epithelial-enriched cell fractions were obtained from the breast milk (combined from both breasts) of 102 women (see Wong et al.[33]). Therefore, all following analyses are based on 134 women for the Biopsy Group and 102 women for the Reference Group.

Of the women who provided milk for the Biopsy Group, the majority (72%) was recruited through the Army of Women (www.armyofwomen.org). The remainder was recruited through various websites and by sending brochures to mammography centers and lactation consultants. The 134 women in the Biopsy Group resided in 36 states including CA (12), CO (2), CT (3), FL (4), GA (5), HI (1), IA (4), IL (10), IN (1), KS (2), KY (1), MA (16), MD (6), ME (1), MI (5), MN (3), MO (1), MT (1), NC (4), ND (1), NE (1), NJ (5), NM (2), NY (8), OH (1), OK (1), OR (1), PA (2), RI (1), SC (2), TN (1), TX (9), VT (1), VA (6), WA (6) and WI (4).

Of the women who provided milk for the Reference Group, the majority (~80%) lived within 20 miles of Amherst, MA and were recruited through local advertisements and fliers. The remaining roughly 20% of women lived within 100 miles of Amherst, MA or were visiting Amherst at the time they donated a milk sample.

Subject demographics. Neither mean age, age at first birth, baby's age at time of milk donation, number of live births, nor

BMI differed significantly between the 134 women in the Biopsy Group and the 102 women in the Reference Group (**Table 2**). The majority of women in both the Biopsy and Reference Groups were Caucasian (88 and 90%, respectively). There was a small percentage of Hispanics (7 and 1%, respectively) followed by Asian and Pacific Islanders (2 and 6%, respectively) and African Americans (2 and 1%, respectively) in each of the groups. The only demographic that differed significantly between the Biopsy and Reference Groups was family history of breast cancer. Thirty percent of the women in the Biopsy Group had a first degree female relative (mother or sister) with breast cancer compared to only 7% in the Reference Group (Fisher's Exact p = 0.000), and 63% of women in the Biopsy Group had any family history of breast cancer compared to 45% in the Reference Group (Fisher's Exact p = 0.006).

Milk sample demographics. The major difference in the breast milk samples between the two study groups was the origin of the milk. In the Reference group, the single milk sample from each woman came from either one breast or both breasts, but this information was not obtained from each woman. In contrast, every milk sample in the Biopsy Group came from one breast only, the origin of the milk (left or right breast) was recorded and all but one woman provided milk samples from both breasts. The mean volumes of the milk samples were similar between the biopsied and non-biopsied breasts from all women in Biopsy Group (59 and 57 mL, respectively) and yielded similar numbers of epithelial-enriched cells, and ng of DNA (**Table 3**). In contrast, the volume of the combined milk samples in the Reference Group were 48% greater (mean = 86 mL) but yielded roughly 100% more epithelial cells and 290% more DNA (**Table 3**). Note that there are differences between Group sample size and demographic sample size in **Table 3**. In several cases the volume of milk was not recorded, the cells in the epithelial-enriched fraction were not counted, and the DNA was not quantified.

To determine if having a recent biopsy altered the sample demographics of the Biopsy Group, we compared the volume of milk, number of epithelial cells and ng of DNA obtained from 30 women who had a biopsy within one year of milk donation to those who had a biopsy greater than one year apart from their milk donation (**Table 3**). There were no significant differences in any of the sample parameters regardless of the time since biopsy or whether the sample was from the biopsied or non-biopsied breast.

Comparison of methods for assessing methylation in the biopsy and reference groups. For both the Biopsy and Reference Groups, the cell separation, DNA extraction, bisulfite treatment and PCR amplification were conducted under similar conditions in our laboratory as described in the methods. The pyrosequencing, however, was conducted in two different laboratories: for the Biopsy Group the sequencing was conducted in our laboratory on a PyroMark Q24, while the pyrosequencing for the Reference Group was conducted in a commercial laboratory on a PSQ96 HS. To address the potential impact of differences in pyrosequencing equipment/methods for Biopsy and Reference Groups, pyrosequencing was repeated for selected samples from the Reference Group using the same equipment used to perform pyrosequencing for the Biopsy Group. In 2011, archived bisulfite-modified DNA from 22 epithelial-enriched cell samples were retrieved from the minus 80°C freezer amplified for *RASSF1*, *SFRP1* and *GSTP1* and products were sequenced on the Pyromark Q24. **Figure 1** shows the similarity of CpG-specific and overall mean methylation scores of two representative samples sequenced on the PyroMark in 2011 and the PSQ96 in 2008. For the 22 samples, the overall mean methylation scores for *RASSF1*, *SFRP1* and *GSTP1* did not differ between samples sequenced in 2011 and the same samples sequenced in 2008 (*RASSF1*: 4.75 (2011) vs. 4.45 (2008); t = 0.64; df = 21; p = 0.53; *SFRP1*: 9.21 (2011) vs. 6.53 (2008); t = 1.41; df = 19; p = 0.09, and *GSTP1*: 3.13 (2011) vs. 3.72 (2008); t = -1.33; df = 11; p = 0.21). Furthermore, an ANOVA revealed no CpG site-specific differences for *RASSF1* between the samples analyzed in 2008 and again in 2011 (F = 0.96; df = 8, 1; p = 0.33). However, there

Table 1. Summary of biopsy results for the 134 women in Category 1

Biopsy types; n (%)		
FNA	14	(10.45)
Core	51	(38.06)
Surgical	63	(47.01)
Unknown	6	(0.04)
Biopsy diagnoses; n (%)		
Adenoma	1	(0.75)
Adenosis	3	(2.24)
Adipose tissue	2	(1.50)
Angiolipoma	1	(0.75)
Cyst	8	(5.97)
Dense stroma	1	(0.75)
Diffuse cystic mastopa	1	(0.75)
Duct ectasia	1	(0.75)
Fat Necrosis	1	(0.75)
Fibrocystic change	13	(9.70)
Fibroadenoma	54	(40.30)
Fibrosis	12	(8.96)
Galactocele	2	(1.50)
Granuloma	1	(0.75)
Haramtoma	1	(0.75)
Hemangioma	1	(0.75)
Inflammatory cyst	1	(0.75)
Inflammatory lymph node	2	(1.50)
Lactating adenoma	2	(1.50)
Lactational changes	14	(10.45)
Lipoma	1	(0.75)
Mastitis	5	(3.73)
Negative	5*	(3.73)
Phyllodes tumor	1	(0.75)

*An additional five women received a negative diagnosis based on FNA, which was considered insufficient to retain them in the low risk Category 1.

Table 2. Percent is based on the number of women from each group who responded

Subject demographics	Biopsy group		Reference group	
Participants; *n*	**134**		**102**	
Age at milk donation; mean, (S.D.) and range in years	34.0 (4.6)	23–52	32.3 (5.6)	19–45
Age at biopsy; mean (S.D.) and range in years	29.7 (6.0)	13–51	NA	NA
Years since biopsy; mean (S.D.) and range in years	4.3 (3.9)	-0.3-15.9	NA	NA
Age at first birth; mean (S.D.) and range in years	30.6 (4.6)	18–52	29.8 (5.2)	19–43
Baby's age at milk donation; mean (S.D) and range in days	277.3 (230.4)	30–1440	246.3 (188.7)	30–900
Number of live births; mean (S.D.) and range	1.9 (0.88)	1–5	1.6 (0.83)	1–5
Body mass index; mean (range)	23.8 (4.1)	18.2–39.3	24.8 (5.6)	16.2–59.3
Race/Ethnicity; n (%)				
Caucasian	118 (88.06)		90 (90)	
Hispanic	9 (6.72)		1 (1)	
Asian and Pacific Islander	3 (2.24)		6 (6)	
African American	3 (2.24)		1 (1)	
Other	1 (.75)		2 (2)	
First degree female relative with breast cancer; n (%)	39 (29.1)		7 (6.7)	
Any family history of breast cancer; n (%)	85 (63.4)		46 (45.1)	

There were missing data from 1–5 women for either age at first birth, baby's age at milk donation, BMI or race/ethnicity; NA, not applicable.

Table 3. Sample demographics

	Reference group (n = 102)		Biopsy group (n = 134)				Women in biopsy group who had a biopsy within one year of donating a milk sample			
			Biopsied breast		Non-biopsied breast		Biopsied breast		Non-biopsied breast	
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
Milk (volume in mL)	102	86 (33)	126	59 (26)	131	57 (28)	28	56 (31)	30	56 (32)
Cell number in epithelial-enriched fraction ($x10^6$)	101	2.5 (3.0)	126	1.2 (3.1)	130	1.4 (5.6)	28	0.9 (2.5)	30	0.7 (1.0)
DNA (ng)	94	2591 (2919)	117	668 (517)	122	661 (622)	27	626 (431)	29	684 (570)

See text for an explanation of differences in sample size.

was a significant difference for *SFRP1* between the samples analyzed in 2008 and again in 2011 (F = 5.04; df = 7, 1; p = 0.025) and for *GSTP1* (F = 8.60; df = 12, 1; p = 0.004). It is possible that the CpG site-specific differences between the two analyses were related to the length of time the bisulfite-modified DNA was archived (over two years) or the number of times the aliquot was thawed. Regardless of the reason for the site-specific differences, we limited all comparisons between the two study groups to the overall mean methylation scores, as these values did not differ between the same samples analyzed in 2008 on the PSQ96 HS and in 2011 on the PyroMark Q24.

Differences in CpG promoter methylation in epithelial cells from the breast milk of women in the biopsy versus the reference group. We examined the methylation levels of 30 individual CpG sites within promoter regions of three tumor suppressor genes. As can be seen in **Figure 2**, the two populations differ in methylation scores. The most dramatic difference between the two populations is the greater number of outlier scores in the Biopsy Group, particularly for *GSTP1*. This might be expected given that the sample sizes in the Biopsy Group (individual breasts) were roughly twice that of the Reference Group (single sample per woman). Therefore we examined the percentage of population outliers by calculating the scores that were greater than the 75th percentile + 1.5*IQR of the combined Biopsy and Reference Groups. The percentage of outlier scores is significantly greater in the Biopsy Group than in the Reference Group for *GSTP1* (16.3 vs. 0%; Fisher's Exact p < 0.00) but not for either *RASSF1* (9.7 vs. 5.9%) or *SFRP1* (7.8 vs. 5.9%). While less dramatic than the outliers, the overall means also are significantly higher in the Biopsy Group than in the Reference Group for both *RASSF1* (7.00 vs. 4.72; t = 2.93; p = 0.002) and *GSTP1* (9.06 vs. 3.64; t = 5.14; p = 0.00), but not for *SFRP1* (6.29 vs. 5.80; t = 0.63; p = 0.27).

Next, to determine the extent to which the differences between the two study groups were related to demographic covariates, we conducted pooled OLS-ANOVA comparisons including study group, age, age at first birth, number of live births, baby's age (as a surrogate for length of nursing), BMI and family history of breast cancer (first degree female relative with breast cancer and any family history of breast cancer were considered separately).

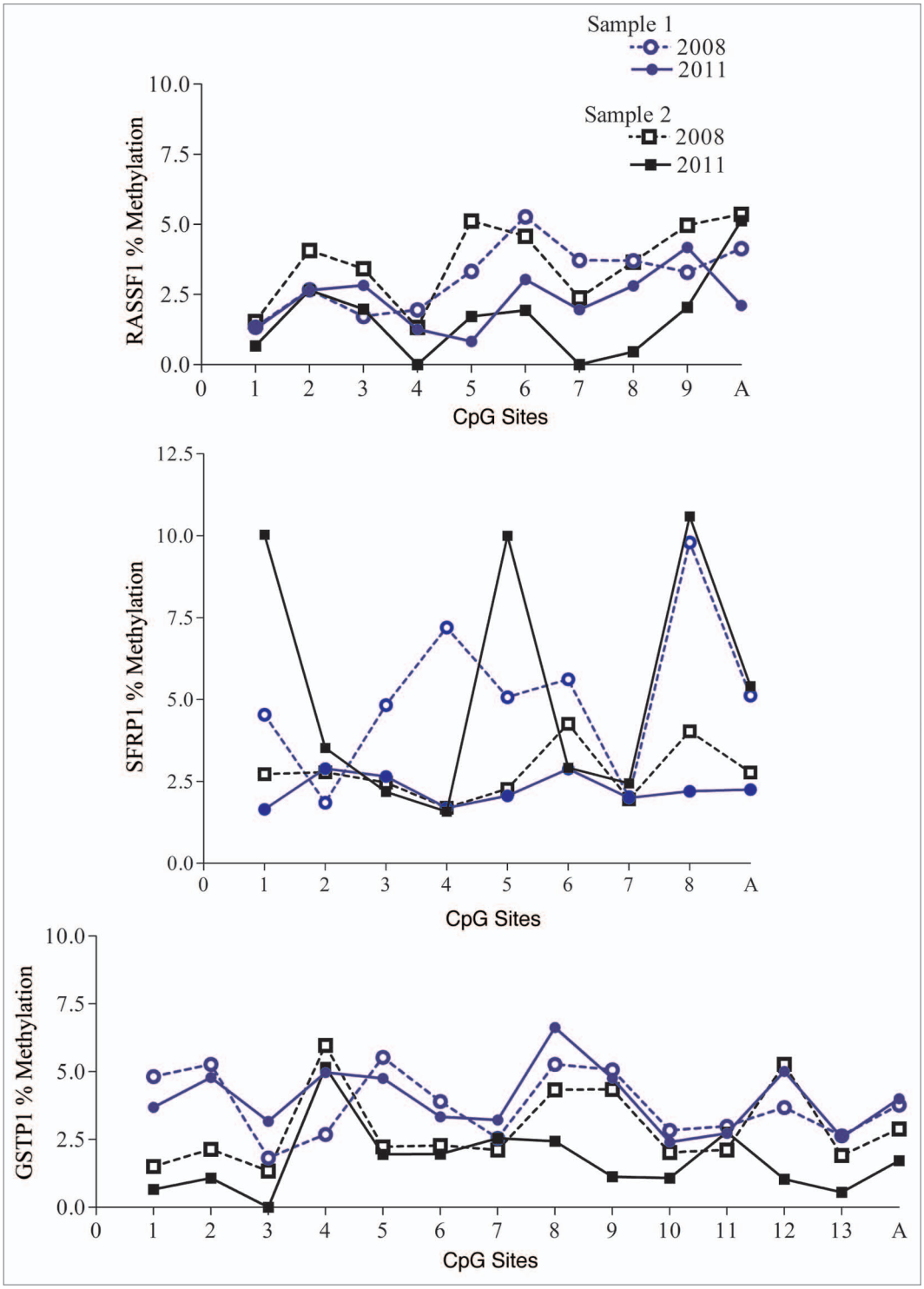

Figure 1. Comparison of the pyrosequencing methods used for methylation analyses in the Biopsy and Reference Groups. Gene-specific PCR amplification and pyrosequencing was performed on a total of 22 archived samples of bisulfite-modified DNA stored for over two years at minus 80°C. Results from two representative samples of bisulfite-modified DNA sequenced in 2008 on the PSQ96 HS and again in 2011 on the PyroMark Q24 are shown for *RASSF1* (top part), *SFRP1* (middle part) and *GSTP1* (bottom part). Filled symbols and solid lines indicate samples sequenced in 2011; open symbols and dashed lines indicate samples sequenced in 2008; A = average of all CpG sites per gene.

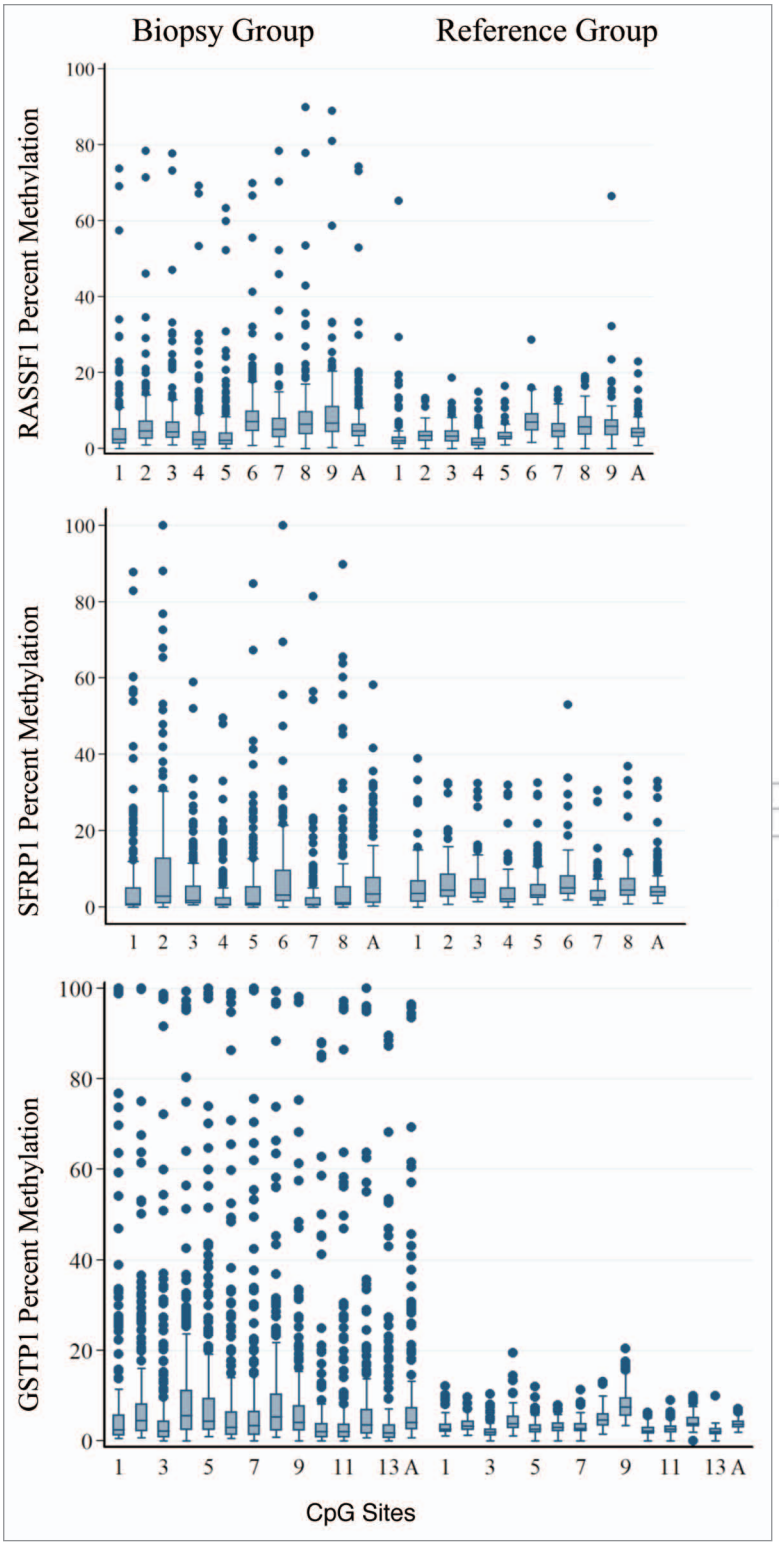

Figure 2. Percent methylation in the Biopsy and Reference Groups. Percent methylation for all samples in the Biopsy Group (134 women; separate samples from each breast) and Reference Group (102 women; single milk sample from one breast or combined from both breasts) are shown for *RASSF1* (top), *SFRP1* (middle) and *GSTP1* (bottom). Box and whisker plots for each CpG site provide the median score (center line), the 25th and 75th percentiles (bottom and top of boxes) and the highest and lowest scores that are within 1.5 times the range of the box. Filled circles represent individual women's scores that were above or below 1.5 times the range based on the combined methylation scores of both the Biopsy and Reference Groups. A = average methylation score for all sites within the CpG island. Sample sizes for the Biopsy and Reference Groups are respectively 184 and 102 for *RASSF1*, 211 and 101 for *SFRP*, and 229 and 99 for *GSTP1*.

Methylation scores were not associated with a woman's age, age at first birth, number of live births, baby's age or her BMI for any of the three genes. However, a lack of any family history was associated with increased methylation for *GSTP1* (t = 2.83; p = 0.005) but not for *RASSF1* and *SFRP1*. Being Caucasian also was associated with slightly higher methylation scores for *GSTP1* (t = 2.12; p = 0.035). By far the major contributor to the observed differences in methylation scores for *RASSF1* and *GSTP1* was study group (t = 2.84; p = 0.005 and t = 4.05; p = 0.000, respectively) suggesting that recruiting women who had breast biopsies resulted in a group with greater mean promoter methylation in exfoliated breast epithelial cells. However, despite the highly significant differences between study groups, only a small percentage of the variability in mean methylation scores is explained by the regressions including study group (*RASSF1*: R^2 = 0.052; *GSTP1*: R^2 = 0.075). Given that little of the variation between the Groups was explained by established risk factors, we next focused on the Biopsy Group to determine whether the methylation signal was specific to the biopsied breast and the extent to which demographic factors explained the variation within the Biopsy Group.

Methylation patterns in biopsied and non-biopsied breasts. Comparisons of the overall and individual CpG methylation means between the biopsied and non-biopsied breasts for all women in the Biopsy Group revealed differences among the three genes (**Table 4**). For *RASSF1* and *SFRP1* the mean methylation scores of each of the individual CpG sites as well as the overall means were higher in the biopsied breast. However, for *RASSF1* only one CpG site, CpG-6, had a significantly higher methylation score in the biopsied breast, while for *SFRP1* the mean scores for CpG1 and CpG5, as well as the overall mean, were significantly higher in the biopsied breast. Likewise, the number of outliers was greater in the biopsied breast for all 9 of the CpG sites in *RASSF1* and 7 of 8 CpG sites in *SFRP1*. In contrast, the individual mean CpG and overall mean methylation scores for *GSTP1* were similar in the biopsied and non-biopsied breasts with the methylation scores for four of the 13 CpG sites slightly higher in the non-biopsied breast. Also, the number of outliers in the biopsied and non-biopsied breasts was similar between breasts, with three of the 13 *GSTP1* CpG sites having a greater number of outliers in the non-biopsied breast. Neither family history of breast cancer nor time between biopsy and milk donation were significantly associated with mean methylation scores for any of the genes.

Table 4. Mean percent methylated DNA for each of the three genes examined in the Biopsy Group

	RASSF1						SFRP1						GSTP1					
	Biopsied breast (n = 98)			Non-biopsied breast (n = 80)			Biopsied breast (n = 106)			Non-biopsied breast (n = 103)			Biopsied breast (n=112)			Non-biopsied breast (n = 113)		
CpG	%M	IQR	#OL	%M	IQR	#OL	%M	IQR	#OL	%M	IQR	#OL	%M	IQR	#OL	%M	IQR	#OL
1	6.24	3.61	12	4.23	4.30	7	**8.0**	5.83	**20**	**4.04**	1.67	**7**	8.58	5.71	17	8.41	3.67	13
2	7.76	4.84	10	6.09	3.86	6	10.61	12.47	7	9.16	10.49	7	10.40	7.55	16	10.49	5.64	13
3	7.82	3.96	12	5.88	4.61	7	4.94	4.14	12	4.71	4.38	11	8.01	4.51	18	7.04	2.94	15
4	5.75	3.32	15	3.84	3.21	9	4.08	2.96	17	2.97	1.40	12	11.60	9.21	15	11.56	8.32	15
5	5.30	3.48	14	3.83	2.80	9	**7.56**	5.91	**15**	**2.92**	2.95	**4**	10.71	7.52	14	10.62	6.01	16
6	**10.17**	4.96	11	**7.92**	5.02	6	8.74	6.68	9	7.18	8.10	6	8.57	5.39	13	9.00	5.38	12
7	8.27	4.91	10	6.21	4.32	5	4.89	3.02	13	2.60	1.12	9	9.81	5.49	14	8.63	4.81	13
8	10.01	6.12	11	7.49	5.40	5	7.64	5.76	12	5.26	4.33	8	11.17	8.66	14	11.02	8.25	11
9	11.20	6.84	9	8.10	6.64	2							10.45	6.98	14	10.12	5.87	11
10													6.87	2.86	11	7.39	3.57	11
11													7.80	2.59	13	8.44	4.26	17
12													9.12	5.46	13	9.19	5.53	10
13													6.73	2.83	12	6.90	2.98	13
Overall	7.90	2.95	13	5.94	3.06	8	**7.46**	8.06	10	**5.09**	5.13	6	9.10	5.00	13	9.04	4.80	12

%M, mean percent methylated DNA. IQR, interquartile range. # OL, number of outliers >(75th percentile + 1.5 x IQR); outliers are computed relative to the overall mean and IQRs for all breasts. **Bold**, Significant ($p < 0.05$) differences in mean scores and/or number of outliers between the biopsied and non-biopsied breasts.

It is important to note that the 62 overall outlier scores, presented by gene and breast in **Table 4**, come from a total of 51 different women or 38% of the 134 women. Only ten women had more than one outlier score (7.5%). Five women had outlier scores in two genes in their biopsied breast (3.7%), three women had outlier scores in two genes in their non-biopsied breast (2.2%), two women had an outlier score for a one gene in each breast (1.5%) and one woman with two outlier scores in her biopsied breast had an additional outlier score in her non-biopsied breast (0.75%) (See **Table S1** for details including missing values).

To assess the relationship between the methylation of epithelial cells obtained from the biopsied and non-biopsied breasts of individual women, we restricted the analyses to those women with a biopsy in only one breast and methylation scores from both breasts. Of the 134 women in the study, five women had biopsies in both breasts, and the samples from an additional ten women yielded no methylation data from one of their breasts. Thus, mean CpG methylation was compared between the biopsied and non-biopsied breasts of the remaining 119 women. For each gene only a subset of the pyrosequencing assays passed inspection resulting in paired sample sizes of 60, 80 and 92 for *RASSF1A*, *SFRP1* and *GSTP1*, respectively. As can be seen in **Figure 3**, the extent to which the mean methylation score in the biopsied breast predicts the mean methylation score in the non-biopsied breast is extremely low for all three genes. However, there is a significant relationship between the mean methylation scores of the biopsied and non-biopsied breasts for *RASSF1* ($p < 0.006$).

Discussion

We previously demonstrated that exfoliated epithelial cells isolated from breast milk can be used to examine DNA promoter methylation of tumor suppressor genes and possibly to assess a young woman's risk of developing breast cancer later in life.[33] However, the degree to which the methylation pattern we observed was an indicator of the overall health of the breast or a signal specific to a lactating breast was uncertain. Comparing the methylation patterns we observed in healthy lactating women at average risk for breast cancer with those from lactating women at potentially increased risk of breast cancer will help us determine whether the DNA methylation pattern in exfoliated cells in breast milk can be used to assess breast cancer risk. To study breast milk from women at increased risk of developing breast cancer, we recruited lactating women who either had, or were scheduled to have a breast biopsy. The vast majority of women who enrolled in the study received a biopsy diagnosis of non-proliferative disease (Category 1), a diagnosis typically associated with little if any increased breast cancer risk. Given the low risk associated with non-proliferative disease, we expected to see a modest increase in promoter methylation and a small subset of the participants with greater methylation. Results presented here are the first to be reported from an ongoing study of methylation patterns in epithelial cells isolated from breast milk that includes long-term follow-up.

Analysis of DNA methylation in the exfoliated epithelial cells isolated from the milk of 134 women with a breast biopsy diagnosis of non-proliferative disease revealed three major findings:

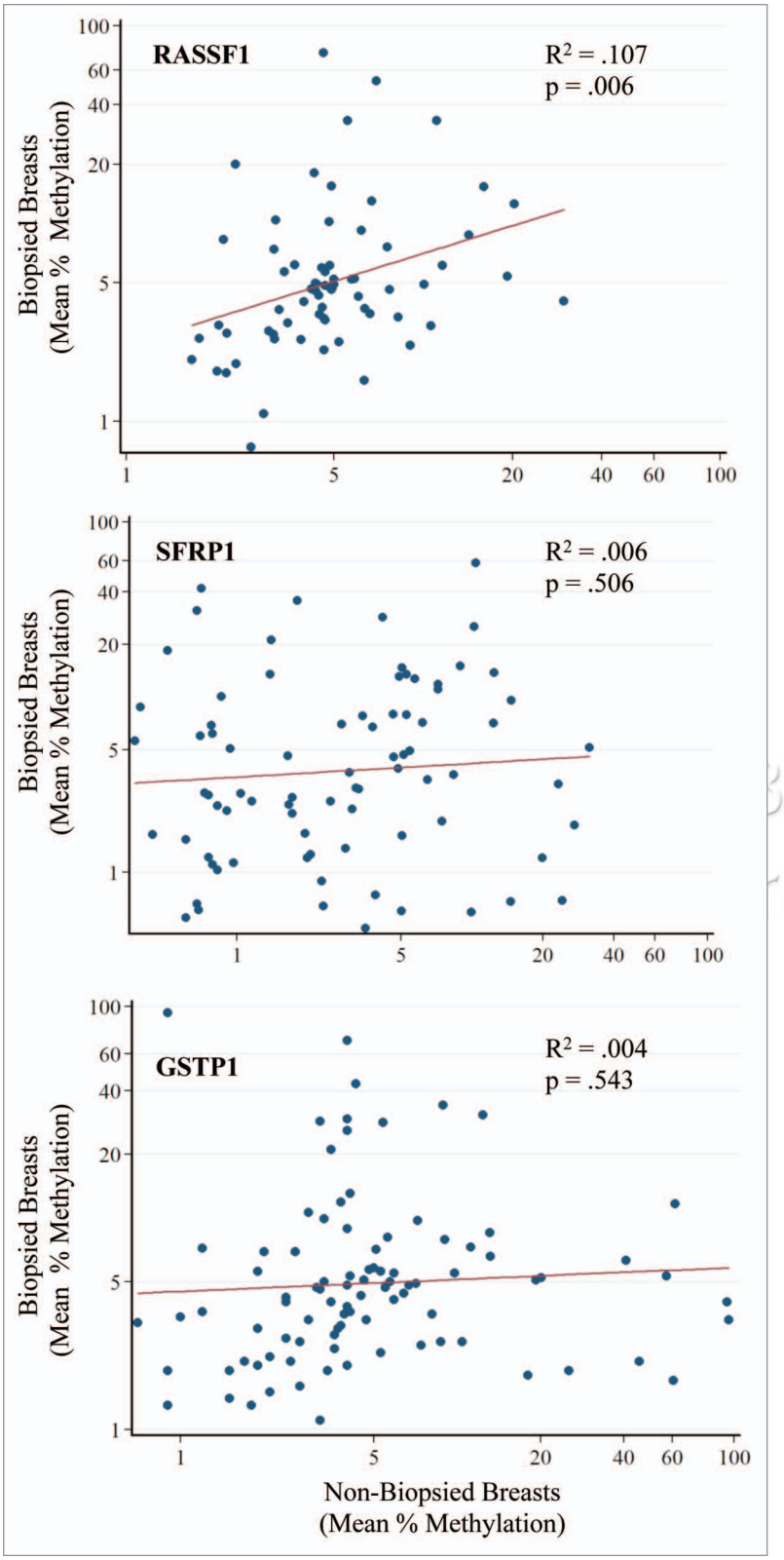

Figure 3. Comparison of biopsied and non-biopsied breasts. The correlation between mean CpG methylation scores of the biopsied and non-biopsied breasts is presented for women who had a biopsy in only one breast and had some methylation data from each breast. Log of mean methylation scores is shown for *RASSF1* (top), *SFRP1* (middle) and *GSTP1* (bottom).

(1) the group of women with a non-proliferative breast biopsy diagnosis had a slightly increased probability of CpG promoter methylation in two of three genes examined as compared to a group for whom a breast biopsy was not a requirement; (2) the methylation patterns differed between the biopsied and non-biopsied breasts for two of the three genes and (3) while the mean methylation scores were low for most women, a subset of women had significantly higher CpG promoter methylation.

The difference in mean methylation scores and percentage of outliers between the Biopsy and Reference Groups is strong evidence that the methylation signal is providing important information about the breast and is not simply indicating the breast is lactating. Both groups of epithelial cells come from the breasts of lactating women of similar ages and reproductive history. The major difference between the groups is the recruitment strategy and selection criteria of a previous biopsy. The women in the Biopsy Group are a self-selected group of nursing mothers residing in 36 different states, the majority of whom responded to Internet recruiting strategies describing a study on molecular markers associated with breast cancer risk. As a study population they are unusual in that 30% report having a mother or sister with breast cancer. This percentage is much higher than the population average of 5–7%,[34,35] suggesting that these women may have been highly motivated to participate in a breast cancer study because of a family history of breast cancer.

While a greater percentage of women in the Biopsy Group had a first-degree female relative with breast cancer, family history was not significantly associated with increased methylation. Indeed, the absence of family history of breast cancer was associated with a significant increase in promoter methylation for *GSTP1*. The lack of correlation between family history and increased methylation is consistent with studies reporting that most breast cancers occur in women without a family history of breast cancer.[7] That little of the variability in methylation scores is explained by established risk factors is expected given the low discriminatory accuracy of most breast cancer risk models.

In normal cells, *RASSF1*, *SFRP1* and *GSTP1* are involved in controlling cell cycle, repairing DNA and metabolizing xenobiotics.[17,25] They were selected for methylation analysis in the present study because they have been shown to be transcriptionally silenced by promoter methylation and are frequently methylated in breast cancer tissue.[18-20,22-24] It is important to keep in mind that the majority of women in the Biopsy Group have low methylation scores. While the overall mean methylation scores are above 5%, greater than 50% of the women are below 5% (overall mean methylation

of 50th percentile for *RASSF1*, *SFRP1* and *GSTP1* equaled 4.65, 3.53 and 4.1, respectively). Furthermore, while the mean values in the Biopsy Group are above those in the Reference Group, the overall mean methylation scores are still very low. More important for assessing personal risk are the outlier scores. While 38% of the 134 women had an outlier methylation score for at least one gene only, 7% had an outlier score in two or more genes. Long-term follow-up on these women should be informative.

In the present study the number of missing values due to failed pyrosequencing among women in the Biopsy Group was high. Data for the biopsied and non-biopsied breasts for each gene were obtained for only a subset of the 134 women (44.8% for *RASSF1*; 59.7% for *SFRP1* and 68.7% for *GSTP1*). This is in stark contrast to the Reference Group in which data were obtained from 97 to 100% of the samples for each gene. The most likely explanation of these differences is the DNA. Nearly four times as much DNA was obtained per sample in the Reference Group; the mean DNA yield of 2.6 μg in the Reference Group allowed 1 μg for the bisulfite conversion. In contrast, the mean DNA yield of 0.671 μg in the Biopsy Group meant that most bisulfite reactions began with substantially less than 1 μg. In general, we have found that conducting the bisulfite reaction with 1 μg DNA results in stronger PCR bands and a higher pyrosequencing pass rate. Additionally, the smaller DNA yield in the present study prevented us from conducting second bisulfite reactions when pyrosequencing failed. Given that a small volume of breast milk can contain millions of exfoliated epithelial cells it is unfortunate that we could not recover more cells from the milk. We isolate the epithelial cells with immunomagnetic beads and hence we must use fresh (never frozen) milk. While all milk samples were sent to our laboratory on ice by overnight express, significant degradation of epithelial cells likely occurred resulting in fewer cells per ml as well as more fragile cells and a lower recovery of DNA. Although the shipment of milk with the resulting delay in processing may have reduced the number of cells and amount of DNA recovered, we think it did not affect the methylation scores. In a small study in which we compared breast milk samples processed immediately after expression with those processed 24 h after expression, we observed no difference in methylation scores (unpublished data).

A major goal of the present and our ongoing study is to select a population in which we can examine the relationship between promoter methylation and breast cancer risk in cells obtained from breast milk. By recruiting women who have had a breast biopsy we are achieving this goal. Our recruitment criteria resulted in a small number of women with outlier scores in more than one gene (7% of the Biopsy Group), an encouraging finding since most women in the Biopsy Group will not develop breast cancer. Long-term follow-up of participants and a greatly expanded part of genes are needed to determine the specificity and precision with which we can use promoter methylation of exfoliated cells in breast milk to predict which women will develop breast cancer. However, this study provides evidence that analyzing promoter methylation of key genes known to be methylated in the early etiology of breast cancer could provide a more individualized measure of risk. While we are using previous biopsy to obtain a high-risk population, analysis of promoter methylation would not need to be restricted to a high-risk population; indeed, it would be most useful in detecting increased risk among women who will develop sporadic breast cancer. Young women of childbearing age are an excellent population for assessing individual risk. These women have already experienced changes associated with critical windows of exposure, yet there is still time to stop and potentially reverse the epigenetic effects associated with many deleterious exposures.

Materials and Methods

Breast biopsy study population. *Subject recruitment and eligibility.* The study was approved by the Institutional Review Boards of the University of Massachusetts and the Congressionally Directed Medical Research Program. We recruited lactating women who either had a breast biopsy or were scheduled to have a breast biopsy to participate in a study of "Molecular Biomarkers for Assessing Breast-Cancer Risk" by advertising on websites and contacting mammography centers, lactation consultants and breast cancer and breastfeeding organizations across the US. Lactating women 18 years or older who either had a breast biopsy or were scheduled to have a breast biopsy were eligible to participate in the study if they were free from debilitating mental and physical illness, and willing to complete a questionnaire, provide a copy of their breast biopsy pathology report and donate a milk sample from one or both breasts. See "Reference Study Population" below for information on the recruitment of women from our published study on methylation in breast milk.[33]

Obtaining and classifying biopsy reports. After donating a milk sample from each breast women were asked to contact their Health Care Provider (HCP) and request a copy of their pathology report. We reviewed each biopsy report and used the classification scheme adopted by the American Cancer Society to classify biopsy results into one of four categories.

(1) Non-proliferative lesions include fibrosis, cysts, mild hyperplasia, adenosis (non-sclerosing), simple fibroadenoma, phyllodes tumor (benign), a single papilloma, fat necrosi, mastitis, duct ectasia, lipoma, hamartoma, hemangioma, hematoma and neurofibroma.

(2) Proliferative lesions without atypia include ductal hyperplasia (without atypia), complex fibroadenoma, sclerosing adenosis, several papillomas or papillomatosis and radial scar.

(3) Proliferative lesions with atypia include atypical ductal hyperplasia, atypical lobular hyperplasia and lobular carcinoma in situ.

(4) Malignant lesions include ductal carcinoma in situ and invasive carcinomas.

Here we present data only from women in category 1, non-proliferative lesions. Although having a breast biopsy is an established breast cancer risk factor, a diagnosis of non-proliferative lesion is not considered to be associated with a significant increase in risk. Most women with this diagnosis will not develop breast cancer. Therefore, we predicted that women in this category would have only a modest increase in promoter methylation as compared to women who did not have a breast biopsy, and that

the higher levels of promoter methylation would be restricted to a small subset of the population.

Collection of breast milk samples. Eligible women were sent an informed consent document and a reproductive history and health questionnaire. Consented women were sent a "breast milk collection box" that included four glass 100 mL bottles, an ice pack, a prepaid return address label and directions for filling the bottles and returning the collection box. All of the bottles were clearly labeled; two bottles were for frozen milk (not used in the present study), and two bottles were for fresh milk from the left and right breasts as indicated on the bottles. Women were asked to place the ice pack and the two bottles for the frozen milk in their freezer at least one night prior to returning the box. The morning they intended to return the box, women were asked to pump or hand express all the milk from each breast, transfer the milk into the appropriately labeled bottles (left fresh and right fresh), place the two bottles with fresh milk along with the frozen milk and ice pack in the collection box, and call the express mail carrier for immediate pick-up. In general, milk samples were delivered to the Arcaro laboratory at the University of Massachusetts by 11:00 AM the following day.

Processing of breast milk. Milk samples were processed upon delivery to the laboratory; usually within 24 h of being expressed. Any frozen milk samples were thawed and transferred to acid-rinsed amber bottles and archived at -20°C for future studies. The fresh (never frozen) milk samples were used in the present study. Five mL of fresh milk from each breast were archived at -20°C in glass vials. The remaining milk from each breast was diluted 1:1 in phosphate buffered saline (PBS) and centrifuged at 1,000 G for 10 min in 50 mL glass centrifuge tubes. Supernatant was removed and stored in 250 mL acid-rinsed amber bottles. Cell pellets were removed and transferred to 50 mL polypropylene tubes, and then washed twice for 5 min at 500 G in sterile PBS. The glass tubes were rinsed with half the original volume of sterile PBS and this rinse was added to the 250 mL acid-rinsed bottles. The diluted milk was then stored at -20°C for future studies.

Isolation of epithelial cells. Washed cell pellets were resuspended in 1 mL of PBS and a cell count was made using a hemocytometer. The epithelial cells were isolated as previously described,[33] using epithelial-specific MACS HEA-125 beads (Miltenyi Biotech, Germany) and a paramagnetic column and stand. Both the epithelial-depleted and epithelial-enriched cell fractions were collected and stored at -20°C until needed. Only epithelial-enriched cell DNA is examined in the current study.

DNA extraction, bisulfite treatment, PCR and pyrosequencing. DNA from the epithelial-enriched cell fraction was extracted using the QIAamp DNA isolation kit as previously described[33] and was quantified and assessed for quality using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington DE). Up to 1 μg of genomic DNA per sample was bisulfite modified using Epitect Bisulfite conversion kit (Qiagen, Valencia CA) as per manufacturer's instructions. PCR primers and sequencing primers are listed in Wong et al.[33] For RASSF1 amplification, 2 μl of bisulfite-converted DNA was amplified in a 25 μl reaction mixture containing 1x PCR buffer, 200 nM primers and 1.25 units of HotStar Taq DNA Polymerase (Qiagen, Valencia CA) over 45 cycles at the specified annealing temperature. For GSTP1 and SFRP1 amplification, a PyroMark PCR kit was used (Qiagen, Valencia CA) as per manufacturer's instructions and at the specified annealing temperature for each gene for 45 cycles. Pyrosequencing was completed using a PyroMark Q24 pyrosequencer (Qiagen, Valencia CA) with 20 μl of PCR product according to the manufacturer's instructions.[36] The methylation status at each CpG site in the target region was analyzed by PyroMark Q24 software version 1.0.10 (Pyrosequencing AB, Uppsala Sweden). Each pyrogram was visually inspected for quality controls including completeness of bisulfite-conversion, expected sequence order and peak height. Data from only those pyrograms that passed all quality controls were included in the analyses. The pass rate was between 75 and 95% per run on the PyroMark Q24.

Reference study population. Although the primary results presented in this paper are from the sample of women with a breast biopsy, it is instructive to compare methylation profiles from the Biopsy Group with those previously reported for 102 women who were not selected for increased risk based on a previous breast biopsy.[33] We will refer to this group as the "Reference" Group and women in the biopsy study as the "Biopsy" Group. There are several differences between the Reference and Biopsy Groups. First, women in the Reference Group were recruited locally from the areas surrounding Amherst, MA, and therefore the milk samples usually were processed within a few hours after being expressed. Second, a single milk sample, from either one or both breasts, was collected from each woman in the Reference Group, and a single cell pellet was obtained from which epithelial cells were isolated and DNA extracted. Third, while methods for the bisulfite-treatment and PCR amplification were identical between the two groups, the pyrosequencing in the Reference Group was conducted by a commercial company (EpigenDx, Worcester, MA) using a PSQ96 HS system (Biotage AB).[33] In contrast, the pyrosequencing for the Biopsy Group was conducted in the Arcaro laboratory as described above using the PyroMark Q24 System (Qiagen).

Statistical methods. Analyses presented include comparisons of both sample demographics and methylation between the Biopsy Group and the Reference Group as well as within subgroups of the biopsy sample, analysis of covariates potentially related to methylation and comparisons between biopsied and non-biopsied breast samples. Methylation and questionnaire data were merged into a combined data file and statistical analyses were performed using STATA 10: Data Analysis & Statistical Software (Statacorp LP, College station, TX). The comparison of pyrosequencing methods between laboratories included both CpG site-specific t-tests and an OLS-ANOVA model for each gene's methylation variance explained by effects of laboratory, CpG site, and the interaction of these main effects. Comparing results across the Reference and Biopsy Groups (e.g., demographics, methylation and covariate effects on methylation) and across subgroups of the biopsy group (e.g., biopsied and non-biopsied breast samples) we employed several different standard statistical tests. For comparison of differences in frequency of enumerative demographic characteristics

between samples or groups we calculated Fisher's exact Phi. For comparison of mean methylation between groups or subgroups we computed independent (unequal variance) sample binomial t-tests. To compare the variance in methylation explained by group and subject characteristics we used both pooled OLS-ANOVA regression models with dummy variable control for sample origin, and replicated independent within-sample regression models. All regression models including cases from the biopsy sample included correction for clustering effects (i.e., multiple observations per woman) on standard errors. To compare the frequency of methylation outliers both between samples and for within-biopsy-sample comparisons of biopsied and non-biopsied breast methylation, we identified methylation outliers (i.e., >75th percentile + 1.5 Pooled Interquartile Range) and compared their frequency across samples or groups using Fisher's exact Phi. All tests were two-tailed and significance was set at 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was supported by grants from The Avon Foundation for Women, CDMRP (W81XWH-08-1-0721) and NSF IGERT (DGE-0654128).

Note:

Supplemental material can be found at:
www.landesbioscience.com/journals/epigenetics/article/18280

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