

# DNA Methylation in Paired Breast Epithelial and White Blood Cells from Women Undergoing Reduction Mammoplasty

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**Abstract.** *Background: The extent to which white blood cell (WBC) DNA methylation provides information on the status of breast epithelial cell DNA is unknown. Patients and Methods: We examined the correlation between methylation in Ras-association domain family-1 gene (RASSF1), a tumor-suppressor gene, and methylation in repetitive elements in paired sets of DNA from WBC and breast epithelial cells collected from 32 women undergoing reduction mammoplasty. Results: We observed no evidence of correlation in methylation levels for ALU, long interspersed nuclear element-1 (LINE1) or juxtacentromeric satellite-2 (SAT2) ( $r=0.02$  for LINE1,  $p=0.98$ ;  $r=0.28$  for ALU,  $p=0.12$ ;  $r=0.26$  for SAT2,  $p=0.17$ ) for matched sets of DNA from WBC and breast epithelial cells. Variability in these markers across individuals and in the same tissue was low. Five women had an average methylation level above 5% for RASSF1 in breast epithelial cell DNA; however, average methylation levels in WBC DNA for these women were all below 1%. Conclusion: Methylation patterns in WBC DNA did not reflect methylation patterns in the breast.*

Epigenetic marks, such as DNA methylation, play an important role in regulating gene expression. Cytosines followed by guanine on the same strand of the DNA backbone are referred to as a CpG site. An important characteristic of a CpG site is that a methyl group can be added to cytosine. A high-density of CpG sites is frequently located within the control region of genes, and methylation in these regions normally serves to silence gene expression. Such CpG islands are also commonly

found in repetitive DNA sequences in the genome. Over one million Alu repeats, a half million long interspersed nuclear elements (LINE1) sequences, as well as other repetitive elements (e.g. adjacent juxtacentromeric satellite 2 (SAT2) in the human genome are normally heavily methylated (1). In these repetitive regions, methylation serves to prevent gene transcription and reduce the likelihood of genetic recombination that could result in harmful mutations. Because these repetitive regions account for over 40% of the methylation in the genome (2), methylation levels in repetitive elements have been used as surrogate markers of genome-wide DNA methylation.

In breast carcinomas, DNA methylation commonly occurs in the promoter regions of tumor-suppressor genes, resulting in deleterious silencing of these cell-regulatory genes (3, 4). Genomic hypomethylation is also commonly observed in breast tumors, and likely contributes to cancer development by promoting genomic instability and the expression of oncogenes (1, 5). It is reasonable to suspect that patterns of DNA methylation in pre-malignant breast tissue may also provide important information on breast cancer risk and early diagnosis. Studying DNA methylation directly in breast tissue is difficult in the clinical setting and in large-scale epidemiological studies; thus, less invasive biomarkers have been investigated. Because white blood cell (WBC) DNA is readily available, there is intense interest in the possibility that WBC DNA methylation levels may serve as a proxy for methylation patterns in breast tissue. Although not well understood, some studies have suggested a possible association between the methylation profile in WBC DNA and the risk of breast cancer (6). As yet, there are limited empirical data on whether WBC DNA methylation levels provide information on the methylation status of breast tissue in the same individual. In the present study, we examined the methylation status of ras-association domain family-1 gene (RASSF1), a commonly methylated tumor-suppressor gene in breast tumors, and three repetitive elements (LINE1, ALU, SAT2) using state of the art pyrosequencing methods in paired breast epithelial cell explants and WBC DNA obtained from a set of women undergoing mammoplasty.

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*Key Words:* Global methylation, LINE1, WBC DNA.

Table I. Sequence data for pyrosequencing assays.

Assay ID	CpG loci	Genomic target sequence	Bisulfite-converted target sequence
<i>RASSF1</i>	Human <i>RASSF1</i> , Exon 1 Ensembl Gene ID: ENSG00000068028 Ensembl transcript ID: ENST00000359365	<b>CGCCCGGCCCGCGCTTGCTAGCGC</b> CCAAAGCCAGCGAAGCACGGGC CCAACCGGGCCATGTCGGGGGA	<b>YGTTTYGGTTYGYGTTTGTAGYG</b> TTTAAAGTTAGYGAAGTAYGGGT TTAATYGGGTATGTGYYGGGGA
<i>LINE1</i>	Human <i>LINE1</i> GenBank Accession#: M80343	<b>CTCGTGGTGCGCCGTTTCTTA</b> AGCCGGTCTGAAAAG	<b>TTYGTGGTGYGTGTTTT</b> TTAAGTYGGTTTGAAAAG
<i>ALU</i>	Human <i>ALU</i> GenBank Accession#: X55933.1	<b>GCCCGCCACTACGCCCGGC</b>	<b>RCCCRCCACTACRCCCR</b>
<i>SAT2</i>	Human <i>SAT2</i> GenBank Accession#: L12216	CATCATCTAATGGAAT <b>CGCATG</b> GAATCATCATCAAATGGAAT <b>CGA</b> ATGGAATCATCATCAAATG	TATTATTTAATGGAAT <b>YGTATG</b> GAATTATTATTAATGGAAT <b>YG</b> AATGGAATTATTATTAATG

Bold letters indicate CpG sites interrogated.

## Patients and Methods

Study subjects were women undergoing reduction mammoplasty at Baystate Medical Center in Springfield, Massachusetts, USA, between 2007 and 2010. The Institutional Review Boards at Baystate Medical Center (Protocol Number: 132304) and University of Massachusetts Amherst (Protocol Number: 2010-0674) approved the study. All participants consented to provide excess tissues from the breast not needed for diagnostic purposes and a blood sample at the time of surgery. The blood was processed immediately and the buffy coat was stored at  $-80^{\circ}\text{C}$ .

Immediately after collection, breast tissues were finely minced and digested overnight in mammary digestion media with collagenase. Any undigested tissue was removed and the digestion solution was centrifuged at  $80 \times g$  for 10 min to collect a mammary epithelial cell pellet. The pellet was then washed in 10 ml cold buffered saline solution and centrifuged, as previously described (7). The pellet was then incubated with 2 mL of trypsin/EDTA for 5 minutes at room temperature, re-washed and pelleted. The pellet was treated with 2 ml dispase and 10  $\mu\text{L}$  DNase I for 5 min at room temperature and then the saline wash and centrifugation was repeated. A single-cell suspension was achieved by passing the digested cells through 100  $\mu\text{m}$  and 40  $\mu\text{m}$  cell strainers and centrifuging for 5 min at  $100 \times g$ . The enriched epithelial cells were cultured in mammo cult media (Stem Cell Technologies Inc. Vancouver, BC, CAN).

Epithelial cell pellets and matched buffy coat specimens were shipped on dry ice from the University of Massachusetts to EpigenDX Inc. (Hopkinton, MA, USA) for methylation analysis. Briefly, 200  $\mu\text{L}$  cell pellets or buffy coat specimens were lysed using Genomic-Lysis Buffer (ZymoResearch, Irvine, CA USA). 20  $\mu\text{L}$  of lysate was directly treated with DNA bisulfite modification reagents (ZymoResearch) and eluted in 18 to 20  $\mu\text{L}$  of buffer. One microliter of bisulfite-modified DNA was used for each gene-specific amplification and 10  $\mu\text{L}$  of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Qiagen Valencia, CA USA). The methylation status of each CpG site was analyzed individually as a T/C SNP using QCpG software (Qiagen). Resulting pyrograms and percent methylation scores for each CpG site were received from EpigenDX, Inc. Each pyrogram was visually inspected for quality controls including bisulfite conversion, expected sequence order and peak height. Data only from those pyrograms that passed all quality controls were included in the analyses. The genomic target sequences

and bisulfite-converted target sequences for *RASSF1*, *LINE1*, *ALU* and *SAT2* are listed in Table I. The mean percentage methylation was obtained by averaging across 9, 4, 4 and 2 CpG sites for *RASSF1*, *LINE1*, *ALU* and *SAT2*, respectively. The mean coefficient of variation (CV) based on three blinded duplicates of buffy coat specimens were 0.5%, 3.9% and 1.5% for *LINE1*, *ALU* and *SAT2*, respectively. The coefficient of variation for *RASSF1* was 15.7%, based on two duplicates that had non-zero percent methylation scores. Spearman correlation coefficients were used to evaluate the pairwise correlation of methylation of *LINE1*, *SAT2*, and *ALU* within a source of DNA and to evaluate the correlation of methylation of each of three markers across DNA marker source.

## Results

The methylation analysis focused on 32 women for whom we had both buffy coat and mammo cult specimens. As shown in Table II, demographic and personal characteristics were available for 22 of these 32 women. The mean age of these participants was 35.1 years, with 41% of the participants being over 40 years of age. Fifty percent were nulliparous and among parous women, only one had a first birth after 24 years of age. Only 5% had a family history of breast cancer. Over 60% were pre-menopausal.

Figure 1 displays boxplots of each methylation measure (*LINE1*, *ALU*, *SAT2*, and *RASSF1*) in breast epithelial cells and WBC DNA. In both DNA from breast epithelial cells and WBC DNA, the mean methylation levels of repetitive elements were highest for *LINE1*, intermediate for *SAT2*, and lowest for *ALU*. As shown in Table III, mean methylation levels in WBC DNA were highest in *LINE1*, intermediate in *SAT2*, and lowest in *ALU*. A similar pattern was observed in breast epithelial cell DNA. The average methylation in *LINE1* was uniformly higher in WBC DNA than in breast epithelial cell DNA. Variability in these markers across individuals in both tissues was strikingly low, as demonstrated by the low coefficient of variations observed: 1.2%, 4.3% and 3.2% for *LINE1*, *SAT2* and *ALU* in WBC DNA and 2.3%, 6.2% and 3.4% for these respective markers in breast epithelial cell DNA.

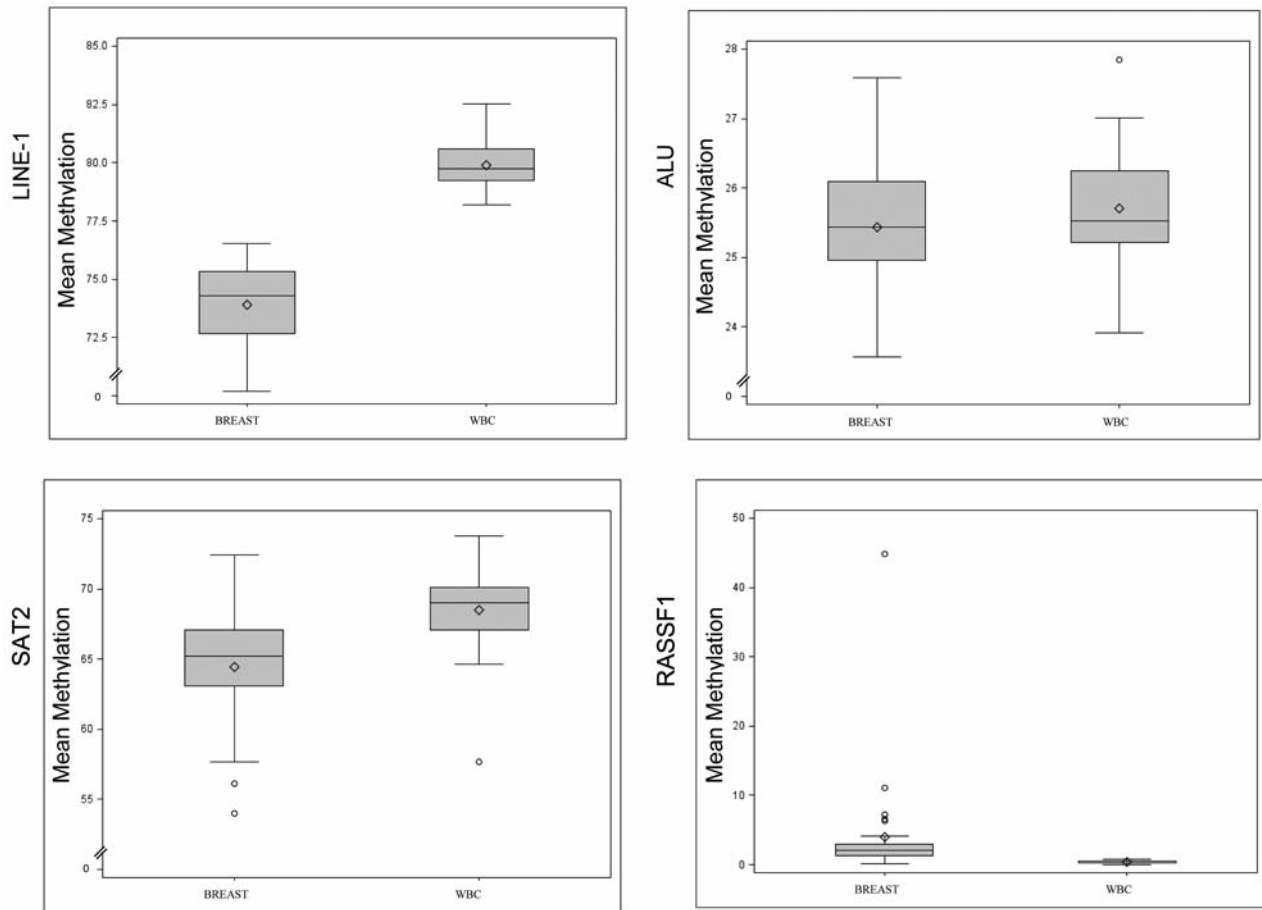


Figure 1. Distribution of DNA methylation levels by specimen type. The box and whisker plots represent the spread of % methylation where the box shows data from the 25 to 75 percentile, and the whiskers show the minimum and maximum excluding outliers that are represented by circles. The line and the diamond in the box represent the median and mean, respectively.

The average methylation level for *RASSF1* was higher in DNA from breast epithelial cells than in WBC DNA (4.0% vs. 0.38%). The coefficient of variation for *RASSF1* across individuals in WBC DNA and breast epithelial DNA was 56.4% and 195%, respectively. However, none of the women in the study had a *RASSF1* methylation level in WBC DNA above 0.73%.

We also examined the pairwise correlation in percentage methylation for the three different repetitive elements within WBC DNA (Figure 2) and within breast epithelial cell DNA (Figure 3). In WBC DNA, there was no correlation between any of three surrogate markers of global methylation ( $r=0.16$ ;  $p=0.39$  for *LINE1* and *ALU*;  $r=0.05$ ;  $p=0.77$  for *LINE1* and *SAT2*; and  $r=0.03$ ;  $p=0.88$  for *ALU* and *SAT2*) (Figure 2). As shown in Figure 3, *ALU* and *SAT2* levels of methylation were moderately positively correlated in breast epithelial cell DNA from the same women ( $r=0.45$ ;  $p=0.01$ ). However, no evidence of a correlation was observed between *LINE1* and *ALU*

methylation levels ( $r=-0.18$ ;  $p=0.33$ ) or between *LINE1* and *SAT2* ( $r=0.26$ ;  $p=0.16$ ) in breast epithelial cell DNA from the same women. There was also no correlation between *RASSF1* and any of the repetitive elements within either WBC DNA or within breast epithelial cell DNA (data not shown).

We next examined the pairwise correlation of the percentage methylation for *RASSF1* and the three repetitive elements in WBC DNA versus that of breast epithelial cell DNA. Correlations were based on 32 women who provided both WBC and breast epithelial cell tissue. As shown in Figure 4, there was no evidence of pairwise tissue correlations between *LINE1*, *ALU* or *SAT2* ( $r=0.02$ ;  $p=0.98$  for *LINE1*,  $r=0.28$ ;  $p=0.12$  for *ALU* and  $r=0.26$ ;  $p=0.17$  for *SAT2*). For *RASSF1*, only five women had hypermethylation, as defined by an average methylation level of 5% or more in their breast epithelial tissue DNA; in one of the women, methylation in *RASSF1* was 44%. None of these five women had mean methylation levels above 1% in their WBC DNA.

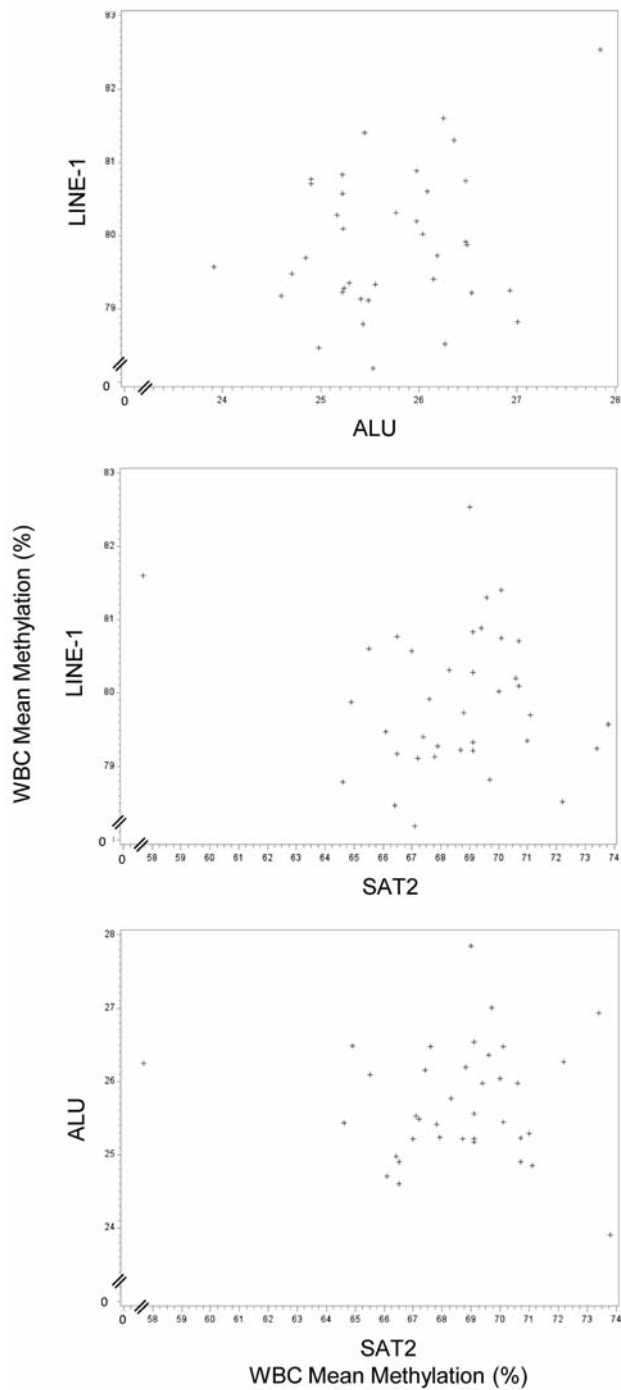


Figure 2. Pairwise correlation in methylation levels of three repetitive elements in white blood cell DNA.

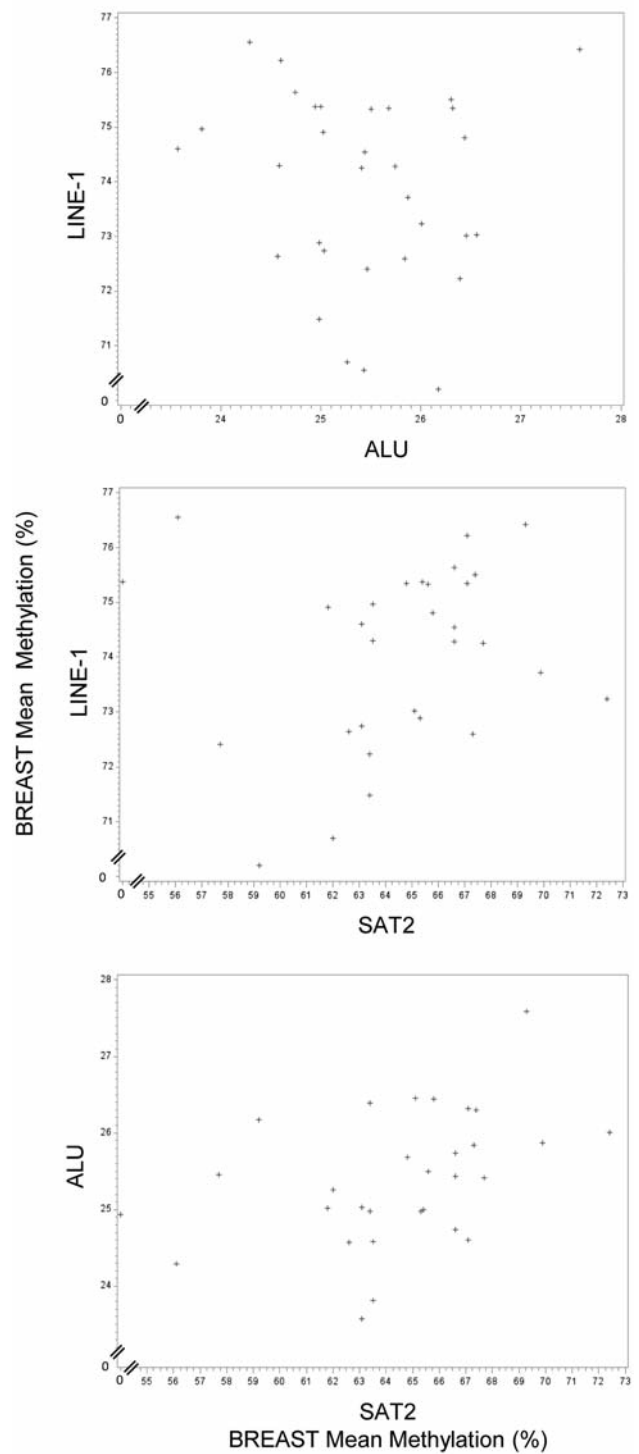


Figure 3. Pairwise correlation in methylation levels of three repetitive elements in breast epithelial cell DNA.

## Discussion

In our study of 32 women undergoing reduction mammoplasty, methylation patterns in *RASSF1*, *LINE1*, *ALU*, *SAT2* in breast epithelial cell DNA were not reflected

in WBC DNA. This finding is not surprising given the very limited variability in these markers across individuals that we observed in both tissues. The fact that the laboratory

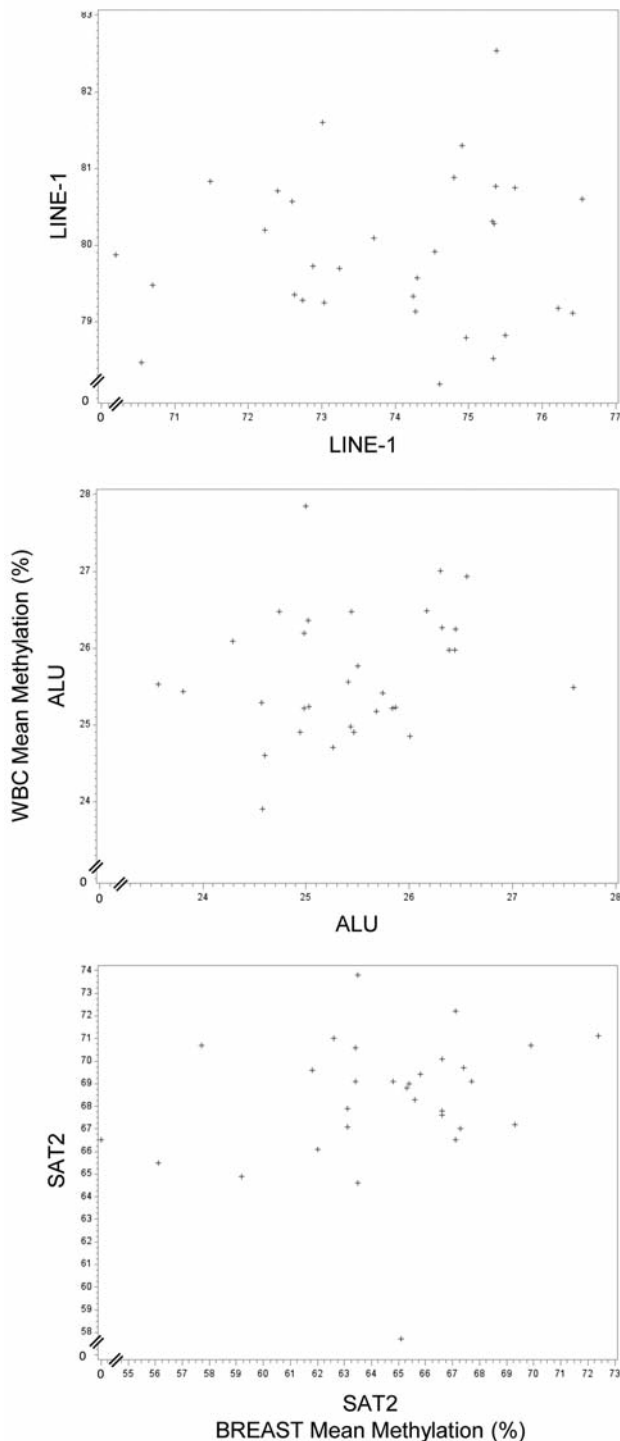


Figure 4. Pairwise correlation in methylation levels of three repetitive elements in matched sets of white blood cell DNA and breast epithelial DNA.

variability was close in magnitude to the limited variability across individuals indicates that mean methylation levels in each tissue were essentially randomly distributed within a

Table II. Study population characteristics of 22 subjects who provided questionnaire data and had both buffy coat and mammo cult specimens.

	Mean (SD)	Range
Age, years	35.7 (15.0)	(16-61)
Body mass index (kg/m <sup>2</sup> )	31.6 (6.0)	(23.0-44.6)
	n	%
Number of live births		
Nulliparous	11	50
1	3	14
2	5	23
3+	3	14
Age at first birth (years)		
<20	4	18
20-24	6	27
25+	1	5
Family history of breast cancer	1	5
Menopausal status		
Pre-menopausal	14	64
Peri-menopausal	3	14
Post-menopausal	4	18
Missing data	1	5

Table III. Mean (SD) percentage methylation of four genes in DNA from leukocytes and breast epithelial cells (n=32).

	Leukocytes Mean (SD)	Breast epithelial cells Mean (SD)
<i>LINE1</i>	79.9 (0.98)	73.9 (1.7)
<i>SAT2</i>	68.4 (3.0)	64.5 (4.0) <sup>‡</sup>
<i>ALU</i>	25.7 (0.83)	25.4 (0.86)
<i>RASSF1</i>	0.39 (0.22)	4.0 (7.8)

<sup>‡</sup>Results on *SAT2* were missing in two cases.

narrow range. To our knowledge, ours is the only study that has examined the correlation in *RASSF1*, *ALU*, *LINE1* and *SAT2* methylation levels in matched sets of breast epithelial cell and WBC DNA in women without known breast cancer. The correlation between methylation levels in these markers was examined in paired sets of WBC DNA and normal tissue adjacent to breast tumor in a prior study of women with breast cancer (8). In that study, there was a positive pairwise correlation in *SAT2* methylation levels between normal tissue adjacent to breast tumor and WBC DNA ( $r=0.67$ ,  $p=0.002$ ). However, the interpretation of this finding is unclear given that the average percentage methylation for *SAT2* in both tissue types was above 100%. Similar to our findings for *ALU* and *LINE1*, however, Cho and colleagues (8) did not observe a correlation for methylation in *ALU* or *LINE1* between WBC DNA and DNA from normal tissue adjacent to breast tumor.



We also did not find a correlation between *RASSF1* methylation levels in WBC DNA and breast epithelial cell DNA. Cho and colleagues reported that two out of two women who had *RASSF1* hypermethylation in WBC DNA also had *RASSF1* hypermethylation in paired normal tissue adjacent to tumor (8), but the interpretation of this finding is limited as the overwhelming majority of women with methylation in normal tissue adjacent to tumor in their study did not have *RASSF1* methylation in their paired WBC DNA specimens. In our study, only five women had *RASSF1* methylation levels above 5% in breast epithelial cell DNA; none of these had evidence of *RASSF1* hypermethylation in WBC DNA. In fact, none of the women in our study had methylation levels in *RASSF1* above 1% in WBC DNA.

In our study, the within-person pairwise correlations were low between the three surrogate markers of global methylation markers in WBC DNA, suggesting that these markers do not rank order individuals similarly. This finding is likely due to the low variance we observed in these markers across individuals in both tissues. Other studies have also noted a low correlation in methylation between *ALU* and *LINE1* in white blood cell DNA. Gao and colleagues (9) reported a correlation of 0.12 between *ALU* and *LINE1* methylation measured by pyrosequencing in WBC DNA in over 350 control subjects. By contrast, Wu and colleagues, in a pooled analysis of 175 breast cancer cases and 228 unaffected sisters, reported statistically significant correlations of 0.39, 0.44 and 0.64 for methylation in *SAT2-LINE1*, *SAT2-ALU*, and *LINE1-ALU* in WBC DNA, respectively (10). These high correlations might be due to the inclusion of women with known breast cancer or to the use of a different method to measure methylation levels. Wu and colleagues recorded more variability in their methylation measurements of the repetitive elements (10), but the interpretation of this variability is complicated by a suggestion, based on a small set of women, that a relatively large proportion of the individuals may have had reported percentage methylation levels above 100%. With the exception of a moderate correlation between methylation levels in *ALU* and *SAT2*, a finding that may have been due to chance as we tested a number of different correlations, we also observed little correlation in methylation of the repetitive elements in breast epithelial cell DNA.

The limitation of the current study is its small sample size. However, our findings provide the first direct information on methylation patterns in several key markers in WBC DNA and breast epithelial tissue in women without cancer. Recently, it has been suggested that observed links between WBC DNA methylation and breast cancer reflect the clonal expansion of leukocytes in response to breast cancer (11, 12). If this were the case, paired associations in methylation levels between WBC DNA and breast epithelial tissue without known breast cancer would not be expected. In summary, the lack of correlation between methylation in *RASSF1* and repetitive elements in

WBC DNA and breast epithelial cell DNA and the low inter-person variability in these biomarkers in both tissues suggests their value as risk indicators of breast cancer will be limited.

## Acknowledgements

The study was supported by a grant from the Baystate Health Foundation-Rays of Hope (principal investigator: SR Sturgeon). Data collection was supported by grants from the National Cancer Institute (R01ES015739: DJ Jerry) and the Avon Foundation (02-2009-01 and 02-2011-028: DJ Jerry and SS Schneider).

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Received March 3, 2014

Revised April 25, 2014

Accepted April 28, 2014