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Quantitative Evaluation of DNA Hypermethylation in Malignant and Benign Breast Tissue and Fluids

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Abstract

The assessment of DNA had demonstrated altered methylation in malignant compared to benign breast tissue. The purpose of our study was to 1) confirm the predictive ability of methylation assessment in breast tissue, and 2) use the genes found to be cancer predictive in tissue to evaluate the diagnostic potential of hypermethylation assessment in nipple aspirate fluid (NAF) and mammary ductoscopic (MD) samples. Quantitative methylation specific (qMS)-PCR was conducted on three specimen sets: 44 malignant (CA) and 34 normal (NL) tissue specimens, 18 matched CA, adjacent normal (ANL) tissue and NAF specimens, and 119 MD specimens. Training and validation tissue sets were analyzed to determine the optimal group of cancer predictive genes for NAF and MD analysis. NAF and MD cytologic review were also performed. Methylation of *CCND-2*, *p16*, *RAR-β* and *RASSF-1a* was significantly more prevalent in tumor than in normal tissue specimens. Receiver operating characteristic curve analysis demonstrated an area under the curve of 0.96. For the 18 matched CA, ANL and NAF specimens, the four predictive genes identified in cancer tissue contained increased methylation in CA vs. ANL tissue; NAF samples had higher methylation than ANL specimens. Methylation frequency was higher in MD specimens from breasts with cancer than benign samples for *p16* and *RASSF-1a*. In summary, 1) routine quantitative DNA methylation assessment in NAF and MD samples is possible, and 2) genes hypermethylated in malignant breast tissue are also altered in matched NAF and in MD samples, and may be useful to assist in early breast cancer detection.

Keywords

methylation; breast cancer; breast fluid; early detection

Introduction

DNA hypermethylation is associated with the development of a variety of human cancers, including breast cancer. Hypermethylation of CpG islands within the promoter region of a gene is recognized as an important epigenetic mechanism of transcriptional silencing of tumor regulatory genes, in many cases tumor suppressor genes, during cancer

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development¹. DNA hypermethylation can be detected in tumor specimens and intraductal samples, including ductal lavage (DL) and nipple aspirate fluid (NAF), from patients with breast cancer^{2,3}. An advantage of these intraductal techniques is that NAF, which is collected from the nipple surface, is noninvasive, and DL and mammary ductoscopy (MD) are minimally invasive since they enter a natural body orifice to collect a sample. MD technology continues to improve. It was recently reported⁴ that a MD available in Europe but not the United States successfully removed through the ductoscope 85% (22/26) of the identified solitary benign tumors.

Alterations in the DNA nucleotide sequence, including loss of heterozygosity and microsatellite instability, can be identified both in tumor tissue and in tissue immediately adjacent to tumor which histologically appears normal⁵⁻¹⁰. Epigenetic aberrations identified in preneoplastic lesions indicate that DNA hypermethylation is frequently an early event in the development of breast cancer^{11, 12}. Levels of cell-free DNA are higher in the body fluids of cancer patients than in healthy controls, with real-time PCR the most sensitive and specific method to identify alterations in DNA methylation¹³. DNA hypermethylation can be detected in a high percentage of NAF³ and DL⁶ specimens which are known to contain malignant cells.

In some of the early studies evaluating NAF and DL, the samples were selected based on cytologic findings, both to correlate methylation changes with cytology, and to optimize the chances of successfully obtaining methylation results. Unfortunately, this approach limits the fraction of samples which can be analyzed, decreasing enthusiasm for methylation assessment as a breast cancer screening tool. We elected to evaluate all samples without cytologic prescreening. Fortunately, cancer-specific DNA methylation patterns have been found in free tumor DNA present in body fluids such as urinary sediment, saliva, sputum, bronchial washings, ejaculate, NAF and DL¹⁴⁻¹⁹. On the other hand, MD provides a more cellular sample, so we analyzed intact cells in these samples.

We routinely collect NAF and MD samples to identify DNA-based biologic markers of breast cancer. Cytologic evaluation of NAF and MD is quite specific but not sufficiently sensitive in detecting breast cancer to be clinically useful alone²⁰. As such, we have investigated DNA based molecular markers to increase the sensitivity of our screening.

Our previous unpublished data as well as data from others^{11, 21-23} using quantitative Methylation-Specific PCR (qMS-PCR) established that *RASSF-1a* and *HIN-1* hypermethylation were present not only in tumor tissue, but also in adjacent histologically normal tissue and/or benign breast tissue. In this study we had three goals: to 1) quantify the methylation level of 10 genes reportedly hypermethylated in breast cancer tissue, and determine those that predicted if breast cancer was present; 2) using the genes found to be predictive, evaluate methylation changes in matched tumor, adjacent normal tissue, and NAF, and thereby determine if methylation changes in NAF were more similar to those in malignant or adjacent normal tissue; and 3) evaluate the diagnostic potential of NAF and MD samples using methylation and cytologic changes. We found that methylation assessment of NAF and MD samples was feasible in a high percentage of samples, and that the results predicted if a breast contained cancer.

Materials and Methods

Patients and Sample Collection

After receiving Institutional Review Board approval, informed consent was obtained from all subjects prior to enrollment. Assays outlined below were run in duplicate.

Tissue samples—Cancer and benign tissue were respectively collected from 44 patients with breast cancer and 34 healthy women who were undergoing breast reduction surgery. Specimens provided by the pathologist immediately after removal from the subject were promptly snap-frozen and stored at -80 °C until use. Serial 10 µM histologic sections were prepared. The first and tenth levels were stained with hematoxylin and eosin for diagnostic purposes. Pathologic diagnosis was verified by light microscopy using levels 1 and 10, and the areas to be microdissected were marked. An average area of 2.5 × 2.5 mm was then microdissected from levels 2 to 9 of the area of interest to obtain a cell population sufficient to secure a detectable band in each PCR reaction. Areas of dissection were chosen to obtain a minimum epithelial cell purity of 90%. Slide microdissection was performed in a laminar flow tissue culture hood. Two µg DNA from each sample was used to evaluate the degree of methylation in 10 genes (*RASSF-1a*, *CCND-2*, *RAR-β*, *p16*, *CST-6*, *GSTP-1*, *E-Cad*, *ER*, *PTEN* and *HIN-1*) demonstrated to be hypermethylated in breast cancer tissue²⁴⁻²⁷.

NAF samples: Assessment of volume required to perform qMS-PCR—NAF volume collected from the breast varies widely, but generally ranges from 10-200 µL. In our quest to identify NAF biomarkers, we aliquot NAF into capillary tubes containing 2-10 µL each, so that multiple biomarkers can be assessed. To determine the NAF volume required for DNA amplification after bisulfite treatment, we conducted experiments using two different NAF volumes and therefore different NAF DNA content. In the first set of experiments, 18 matched breast cancer and adjacent histologically normal tissue samples were obtained at the time of surgery. Matched NAF (15-20 µL) samples were obtained from the same subjects prior to surgery using a modified breast pump as described previously²⁰. Tumor and adjacent normal tissue was microdissected as outlined above.

In the second set of experiments, we determined if a lesser volume of NAF (3-5 µL) would be sufficient to perform qMS-PCR. If successful, a greater fraction of the collected NAF could be used for other biomarker analyses. NAF samples were collected from 106 women, 61 with and 45 without breast cancer. For these samples, matched tissue was not available.

MD samples—148 MD samples were collected as previously described²⁸. All samples came from women who underwent surgery to exclude breast cancer. Each sample was divided into two parts, one for cytologic review and the other for DNA isolation. MD cytologic assessment was carried out as previously reported²⁸. The sample fraction for DNA isolation was spun down at 950 rpm for 10 minutes and the pellet stored at -80 °C until DNA preparation. qMS-PCR analysis was conducted without knowledge of the cytologic findings.

Blood samples—DNA isolated from whole blood obtained from three subjects without breast cancer was pooled and used to produce a standard curve for the unmethylated DNA fraction in performing qMS-PCR.

DNA Isolation and Sodium Bisulfite Treatment

DNA was extracted from malignant and adjacent normal tissue from breasts with cancer and from breast tissue from healthy subjects using proteinase K for digestion followed by heat inactivation at 95°C, then phenol-chloroform and ethanol precipitation. The DNA was dissolved in Tris ethylenediamine tetraacetic acid buffer. A Qiagen Mini-Blood Kit (Qiagen, Valencia, CA) was used to isolate DNA from blood, MD and NAF samples. DNA was isolated from white blood cells as outlined in the kit protocol. For the other samples, the MD cell pellet was suspended in and NAF diluted with 200 µL of PBS and DNA then extracted following the kit protocol. The EZ DNA methylation kit A (Zymo research, Orange, CA) was used for the sodium bisulphite conversion of DNA samples. Briefly, one µg of universal

methyated DNA (Chemicon International, Temecula, CA) and the DNA samples extracted from the tumor, adjacent normal tissue and blood were treated with sodium bisulphite. For all NAF and MD samples where the DNA quantity was lower than 500 ng, one µg salmon sperm carrier DNA was added and the sodium bisulphite treatment carried out to convert unmethylated cytosine residues to uracil, leaving methylated cytosine residues unchanged. The converted DNA was stored at -80 °C until PCR amplification.

First Round Multiplex PCR

Two-step qMS-PCR was employed to determine the fraction of DNA that was methylated in a given sample for each of the following 10 genes: *p16*, *RAR-β*, *RASSF-1A*, *CCND-2*, *CST-6*, *GSTP-1*, *PTEN*, *E-cad*, *HIN-1*, and *ER*. Initial multiplex conventional PCR (two PCR reactions for each sample, where each reaction contains 5 primer sets) was achieved with the AmpliTaq Gold PCR kit from Applied Biosystems (Foster City, CA), and by the universal (outer) primer sets that targeted both methylated and unmethylated DNA at the same loci to obtain the fragment which was then used as template in the second round PCR for all 10 markers. The sodium bisulfite treated DNA was added to the PCR reaction buffer consisting of 0.2 mM dNTP, 15 mM Tri-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 2.5 units of AmpliTaq Gold and 200 mM of each of the forward and reverse primers for the five genes. Programming conditions were 95 °C for 10 min, followed by 35 cycles of 95 °C for 35 s, 55 °C for 40 s, and 72 °C for 45 s, with a final extension cycle at 72 °C for 10 min. A water control was included for the first run PCR reactions to ensure that the samples rather than artifact were being amplified. The yield of the first round PCR products was verified from agarose gels after electrophoresis and staining. Methylation positive and negative DNA and no template controls were included along with the samples being amplified. Primer sequences for the multiplex amplification are summarized in Table 1. The PCR products were stored at -20 °C until use.

Second Round qMS-PCR

First round PCR products were diluted 1:50 to 1:1000 with water depending on the gene to be analyzed. The diluted PCR products (5-10 µl) were amplified with specific gene primers annealing to both methylated and unmethylated DNA using iQ™ SYBR Green Supermix (BIO-RAD, Hercules, CA) following the manufacturer's recommendations, and using a MyiQ single color real-time PCR instrument (BIO-RAD) for 40 cycles. Water control from the first run PCR and a second control were included to rule out nonspecific amplification during second round qMS-PCR. We also included three qMS-PCR reactions that were set up with mixed methylated: unmethylated copies at percentages 4%, 1% and 0.25% to assess amplification rates with different levels of DNA methylation. After PCR amplification, a melting curve test was performed to ensure that a single gene peak was observed for each sample that generated an informational PCR result. A serial dilution prepared from the universal methylated positive DNA and DNA prepared from whole blood were used to generate standard curves, and the level of methylated and unmethylated targets in each sample was calculated against the standard curves. The relative amount of methylation in the sample was calculated using the following formula: % methylation = $100 \times [\text{methylated DNA} / (\text{methylated DNA} + \text{unmethylated DNA})]$ (23).

Statistical Analysis and Graphical Representation of Data

Tissue data from 44 tumor and 34 benign breasts was obtained in order to determine the optimal subset of a set of ten identified genes relative to predicting cancer. Means and medians were calculated for all of the continuous variables. The Wilcoxon Rank Sum test was used to determine if a specific gene was significantly different between the cancer and benign groups. So as to not miss an important gene the alpha level used for each of these tests was 0.15. Once the set of significant genes was identified, logistic regression models

were estimated using these genes as independent variables. When the best model was identified, we then proceeded to validate it. If our sample size had been large enough we would have split the sample in half and used one half to estimate the model and the other half to validate it. Since we couldn't do this, we employed a cross validation method to validate our model. The method employed is identified as the leave-one-out method²⁹. This method sets aside an observation, estimates the model using the rest of the data and then uses the estimated model to predict the value for the data left out. This process is repeated for each of the observations, and is discussed in detail elsewhere. We then used the Wilcoxon Signed Ranks test to compare the predicted set of values to the set of values which were deleted. Additional variables such as menopausal status were added to the models to determine if better predictive models would result. We generated a receiver operating characteristic (ROC) curve which generated optimal cutoff values to define hypermethylation, maximizing the sum of sensitivity and specificity in the prediction of cancer³⁰. The optimal cutoff was above the 90th percentile for methylation values in normal tissue. Cutoff values have been used to distinguish breast cancer cases from controls for the methylation levels in plasma of four genes³⁰.

To determine which subset of six identified genes were the best predictors of cancer, for MD samples we employed statistical methods similar to those outlined above for tissue. For the 18 matched tissue (cancer and adjacent normal) and NAF samples, the Wilcoxon Signed Ranks test was used to determine which of the six genes were significantly different when comparing tumor to adjacent normal tissue, tumor tissue to NAF or NAF to adjacent normal tissue.

Results

DNA methylation in tumor and normal tissue

The percentage of methylated DNA was significantly higher in 44 malignant specimens compared to 34 reduction mammoplasty specimens (Table 2, Figure 1A) for *CCND-2*, *p16*, *RAR-β* and *RASSF-1a*, significantly lower in cancer specimens for *E-Cad* and *ER*. The median age of subjects with cancer was 54, and for those who underwent reduction mammoplasty 38. It has previously been reported that gene methylation increases with age in benign breast epithelium³¹. Because the age of the normal subjects was lower on average than the cancer subjects, we reanalyzed the methylation results after controlling for age, and found that *CCND2* (p=0.01), *p16* (p=0.0001), *RAR-β* (p=0.02) and *RASSF-1a* (p=0.0001) and methylation levels remained significantly higher in malignant breast tissue than in breast tissue from normal subjects. The mean percent of methylated DNA in tumor tissue exceeded 45% for *RASSF-1a*, 8% for *CCND-2* and 6% for *RAR-β*, compared to 2%, 0.2% and 0.2%, respectively, for normal tissue. Indeed, 45-80% of tumor samples (depending on the gene) had levels of methylation higher than 90% of corresponding normal tissue samples (Table 2B). The percent of methylated *PTEN*, *GSTP-1* and *CST-6* was not significantly different in the cancer and normal samples. We were unable to amplify *HIN-1* using a published primer set, and a second set of primers which we generated yielded unreliable results. We evaluated the optimal cutoff for predicting cancer, including methylation levels above the 90th and 100th percentiles of normal tissue, and found that the 90th percentile provided the best discrimination. We generated an ROC curve, considering methylation levels above the 90th percentile for normal tissue used to define hypermethylation⁶. The optimal ROC curve included methylation levels of *CCND2*, *p16*, *RAR-β* and *RASSF-1a*, and clinical variables age and menopausal status, with an area under the curve (AUC) of 0.96 (Figure 2A).

DNA methylation in 18 matched tumor, adjacent normal tissue and NAF samples

In 18 tumor, adjacent normal tissue and NAF samples we analyzed the four genes (*CCND2*, *p16*, *RAR-β* and *RASSF-1a*) found to have a higher methylation frequency in cancer than normal tissue. Our first task was to identify the NAF volume required to obtain an optimal signal. We screened sample with either low NAF volume (3-5 μL) or higher volume (16-20 μL). Only the higher volume provided reliable results. Using the higher NAF volume, all 18 matched tissue and NAF samples from cancer containing breasts provided satisfactory PCR signals for all four genes. As summarized in Table 3A,B and illustrated in Figure 1B, there was increased methylation in all four genes analyzed in tumor compared to adjacent normal tissues. We next determined if methylation levels in NAF were more similar to tumor or to adjacent normal or true normal tissues. Methylation frequency was significantly higher in NAF than adjacent normal tissue ($p=0.0001$) for *p16*, marginally higher ($p=0.067$) for *RAR-β*, and not different for *CCND-2* and *RASSF-1a* (Table 3B).

DNA hypermethylation in MD samples from cancer containing breasts

Median age in MD samples for subjects with cancer was 60, and for subjects without 47. Fifty-seven of 61 subjects with cancer had early (Stage 0-2) disease. Of 148 MD samples, 119 (80%), including 58/76 (76%) from breasts without and 61/72 (85%) from breasts with cancer, provided adequate DNA for PCR amplification of *CCND-2*, *p16*, *RAR-β* and *RASSF-1a*. Of the 119 with adequate DNA, 61 (51%) were from cancer containing breasts and 58 (49%) from breasts without cancer. In Table 4A we report which genes had different methylation levels in cancer (ductal carcinoma *in situ* and invasive breast cancer) compared to precancer (atypical hyperplasia and lobular carcinoma *in situ*) and benign (all other noncancer diagnoses) MD samples. The percent methylated DNA in the MD samples from cancer containing breasts was significantly greater than in breasts without cancer for *p16* and *RASSF-1a*. Notably, *p16* methylation levels were essentially equivalent in precancer and cancer containing breasts and higher than benign breasts, whereas *RASSF-1a* methylation levels in breasts with precancer were intermediate between breasts with cancer and benign breasts. Indeed, 15-34% of tumor samples (depending on the gene) had levels of methylation higher than 90% of corresponding benign MD samples, and 3-26% of tumor samples has methylation levels higher than all corresponding benign samples (Table 4B, Figure 1C). We generated an ROC curve, with methylation levels above the 90th percentile from benign MD samples used to define hypermethylation. The optimal ROC curve included methylation levels of *CCND2*, *p16*, *RAR-β* and *RASSF-1a*, and clinical variables age and menopausal status. The AUC was 84% (Figure 2B).

DNA hypermethylation in lower volume NAF samples

We analyzed 106 specimens (45 from subjects with breast cancer and 61 from subjects without cancer) containing 3-5 μL NAF each. The qMS-PCR signal was weak and we felt unreliable in both the malignant and benign specimens.

Cytology in NAF and MD samples

Cytologic review was performed on 117 MD and 18 NAF samples that were also collected for methylation analysis. Malignant cytology was detected in 0/18 NAF samples from breasts with invasive cancer, 1/11 MD samples from breasts with DCIS and 0/49 samples from breasts with invasive cancer.

Discussion

In this study we evaluated the DNA methylation frequency of 10 genes reported to be methylated in breast cancer. Both because DNA methylation is present in benign breast

epithelial cells, and to avoid the bias generated using conventional PCR, we performed quantitative methylation-specific PCR, which has been reported to double the detection of cancer in DL samples compared to cytology². Our strategy was to test and validate the ability of the 10 genes to predict if a breast contained cancer, then assess the usefulness of the genes which differentiated cancer from normal tissue in two types of intraductal samples, NAF and MD. The strength of our data is its large sample size and multiple sample types, including matched samples, which allow comparison of methylation frequencies in tumor, adjacent normal tissue and intraductal samples, the latter of which are most desirable for early detection of disease. Additionally, we determined if qMS-PCR and cytologic findings could be combined to maximize tumor detection.

Six of the 10 genes tested had altered (four higher, two lower) methylation frequencies in tumor compared to normal tissue. The actual differences in mean and median methylation in *E-cad* and *ER*, the two genes with lower methylation frequencies in cancer compared to normal tissue, were less than one percent and of questionable significance. Our tissue samples were arbitrarily divided into testing and validation sets, with each set yielding similar results. ROC curve analysis demonstrated that the four genes in combination with clinical variables that best discriminated cancer from normal were very accurate in determining if a breast contained cancer. With these encouraging results, we investigated NAF and MD samples, which can be collected in a non- or minimally invasive manner and therefore are potentially useful for breast cancer screening.

In a previous study using conventional MS-PCR, we detected methylation in both cytologically benign and malignant NAF samples from breasts with cancer³. Only NAF samples with adequate epithelial cells for cytologic review (10 or more epithelial cells on a slide, with 10 slides prepared per specimen) were evaluated. Given that 30-60% of NAF samples²⁰ have fewer cells than this, and that in the current study NAF cytologic findings had very low sensitivity, we assessed if qMS-PCR could detect methylation in NAF samples without cytologic prescreening.

A similar question arises regarding the ability to assess samples using minimally invasive techniques such as DL and MD. A recent report³⁴ documents the performance of qMS-PCR in DL samples from women with and without breast cancer. The authors obtained methylation data in 46% of samples from breasts with, and from 60% of breasts without cancer. These authors did not find that cytologic marked atypia or methylation improved the discrimination between breasts with and without cancer. In our study using MD, we were able to obtain methylation data in 76% of breasts without and 84% of breasts with cancer. Similar to the published report, cytology did not improve cancer discrimination, but contrary to the study, methylation levels of *p16* and *RASSF-1a* were significantly higher in MD samples from cancer containing than non cancer containing breasts, and for a given marker, 3-25% of the MD samples from cancer containing breasts had higher methylation levels than any benign sample for the same marker. As such, a subject without known cancer with MD methylation above the maximum for benign subjects may be at increased breast cancer risk. It is useful to know how early in the process of carcinogenesis that methylation frequency changes, especially if one wishes prevent rather than detect cancer. We therefore divided MD samples into three groups (cancer vs. precancer vs. benign), and found that *p16* methylation trended higher in precancer than in benign samples, with MD precancer and cancer samples having similar *p16* methylation levels. While the trend was not significant, it is possible that the precancer sample size limited our ability to detect a significant difference between the groups.

As stated in the Introduction, the second goal of the study was to evaluate methylation changes in matched tissue and NAF, in order to determine if methylation values in NAF

were more similar to those in malignant or adjacent normal tissue. We analyzed the same four genes in which we identified a higher level of methylation in cancer compared to normal tissue in our initial screen of 78 tissue samples from women with and without breast cancer. Methylation frequency was significantly higher in NAF than adjacent normal tissue for *p16* and marginally higher for *RAR-β*, suggesting that the assessment of *p16* and perhaps *RAR-β* methylation levels in NAF may provide information on whether a breast contains cancer. This could be tested in a future study evaluating NAF in women with and without breast cancer.

In general, the % methylation in samples from breasts with cancer was lower in MD than NAF samples. We learned early in the study the optimal NAF volume for methylation analysis and this volume was used for results in Table 3 and Figure 1B. On the other hand, we divided our MD samples in two, half for cytology and half for methylation studies, since we wanted information on both. Unfortunately, cytology has very low sensitivity in detecting cancer, and not all (80%) of the MD samples provided sufficient DNA for qMS-PCR results. Future studies should use the entire MD sample for molecular analyses. Nonetheless, *p16* (and *RASSF-1a*) methylation was significantly higher in MD cancer than benign samples, similar to methylation findings in NAF and in tissue. Moreover, the increase in *p16* methylation appears to occur in precancer, prior to the development of cancer, which is useful if *p16* methylation assessment were to be tested as a screening tool.

In conclusion, we observed that: 1) quantitative assessment of DNA methylation can be performed in the vast majority NAF and MD samples without prior knowledge of the cytologic profile of the samples, although sufficient NAF volume is critical, and 2) genes hypermethylated in malignant breast tissue are also altered in matched NAF and in MD samples, and may be useful to assist in early breast cancer detection.

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Abbreviations used

ANL	adjacent normal tissue
CA	malignant tissue
DL	ductal lavage
MD	mammary ductoscopy
NAF	nipple aspirate fluid
NL	normal tissue
qMS-PCR	Methylation-Specific PCR
ROC	receiver operating characteristic

Statement of Novelty and Clinical Impact

Early breast cancer detection leads to higher cure rates than when disease is diagnosed at a more advanced stage. Imaging studies detect breast cancer earlier than physical examination alone, but still miss many early stage breast cancers and require additional assessment of cells or tissue collected from the breast.

The purpose of this study was to identify cancer predictive genes in breast tissue, and use these genes to evaluate the diagnostic potential of hypermethylation assessment using quantitative Methylation-Specific PCR (qMS-PCR) of breast specific bodily fluid samples, one of which is noninvasive (nipple aspirate fluid, NAF) and the other of which is minimally invasive (mammary ductoscopy, MD). Our testing and validation samples provided a highly predictive set of genes methylated at a higher rate in cancer than normal breast tissue. In cancer containing breasts, *p16* methylation frequency was significantly higher in NAF compared to adjacent normal tissue. *p16* and *RASSF-1a* methylation frequency was significantly higher in MD samples from cancer containing compared to benign breasts. Methylation assessment of NAF and MD may provide a non-and minimally invasive tool to assist in early breast cancer detection.

Figure 1. Plot of raw data for percent methylated DNA in malignant and normal breast tissue. Methylation values for all four genes (*CCND-2*, *p16*, *RAR-β*, and *RASSF-1a*) were significantly higher in malignant compared to normal breast tissue.

Figure 2. Plot of raw data for percent methylated DNA in matched malignant and adjacent normal tissue and NAF. The percent methylation for *CCND-2*, *p16*, *RAR-β*, and *RASSF-1a* were significantly higher in malignant than in adjacent normal tissue. *p16* methylation was higher in NAF than adjacent normal tissue, marginally higher for *RAR-β*, and not different for *CCND-2* and *RASSF-1a*.

Figure 3. Plot of raw data for percent methylated DNA in MD samples from breasts with cancer and benign breasts. Methylation values were significantly higher in breasts with cancer for *p16* and *RASSF-1a*, but not for *CCND-2* or *RAR-β*.

Figure 4.

ROC curves were generated to illustrate the ability of the biomarkers to predict if a woman has breast cancer. **(A)** represents the curve evaluating methylation and clinical variables in tissue samples from 44 patients with breast cancer and 34 healthy women who underwent breast reduction surgery. The AUC was 0.96. **(B)** represents the curve incorporating methylation and clinical variables in MD samples from 61 women with breast cancer and 48 women with benign biopsy results. The AUC was 0.84.

Table 1
Primer Sequences (F: forward, R: reverse) of the 10 Genes Studied

Genes	Outer primer	Methylated primer	Unmethylated primer	References
<i>RASSF1A</i>	F--GTTTAGTTTGGAATTTTGGGGGAG	F--GGGTTTCGTTTGTGGTTTCGTTC	F--GGGGTTTGTTTTGTGGTTTGTGTT	(25)
	R--CCRCAACTCAATAAACTCAAACTC	R--TAAACCCGATTAAACCCGTACTTCG	R--AAACATAACCCAAATTAAACCCATACTTCA	
<i>CCND2</i>	F--TATTTTGTAAAGATAGTTTGTATTTAAAGG	F--GGCGGATTTTATCGTAGTCG	F--AGAGTATGTGTTAGGGTTGATT	(24, 25)
	R--TTTCCCRRAAAACATAAAACCTCC	R--CTCCACGCTCGA TCCTTCG	R--ACATCCTCACCA ACCTCCA	
<i>HIN-1¹</i>	F--AGTGAGGATATTTAGAGAAATTTAGG	F--GCGTCGAGGTTAGTTTCG	F--GTGTTGAGGTTAGTTTGAAGA	(26)
	R--ATCCCTACCTCTAATCCCAA	R--GTAAACGCTTCTAGCCCTAA	R--TAAACACCTTCTACACCTAAAA	
<i>p16</i>	F--AGAAAGAGGAGGGGTTGGTTGG	F--TTATTAGAGGTTGGGCGGATCGC	F--TTATTAGAGGTTGGGTTGGATTGT	(26)
	R--ACRCCRCACCTCTCTACC	R--GACCCCGAACCGCGACCGTAA	R--C A C CCCAAACCAACCATAA	
<i>PTEN</i>	F--GAGGAGTGGTATTAGTTGG	F--GTTTGGGGATTTTTCGTC	F--TATTAGTTTGGGGATTTTTCGTC	(25)
	R--CAAAACAACRAAAACCCCAACCCCTT	R--AAACCTTCTACGCCGCG	R--CCCAACCTTCTACACCA	
<i>CST-6</i>	F--GTTTGTGGGTTTGTGAAAT	F--TCGAGTTTCGTTTGTAGTTTAGGTC	F--TGAGTTTGTGTTTGTAGTTTGT	(23)
	R--AATACYACCAAAACCAACCCA	R--CATAACCGTCAATACCGTCG	R--CCATAACCATCAATACCATCAA	
<i>GSTP-1</i>	F--GGGATTTTAGGGYGTTCCTTTTG	F--TTCGGGTTGAGCGGTCGTC	F--GATGTTTGGGTTGAGTGGTTGTT	(26)
	R--ACCTCCRAACCTTATAAAATAATCC	R--GCCCCAATACTAAATCACGACG	R--CCACCCCAATACTAAATCAACA	
<i>ER</i>	F--GYGAGGTGTTTGGATAGTAGTAAGTT	F--ACGAGTTTACGTCGGGTC	F--TGTGTTTATGAGTTTAAATGTTGGTT	(26)
	R--RAACTCRAAAACACRCTATTAATAAATA	R--ACCCCCCAACCCGTTAAAC	R--AAAAAAACCCCCCAACCAAT	
<i>E-Cad</i>	F--GTGTTTGGGGTTTATTTGGTTGT	F--TGTAGTTACGTAATTTTATTTAGTGGCGTC	F--TGGTTGTAGTTATGTTTATTTTATGTTGTT	(26)
	R--TACRACTCCAAAAACCCATAACTAACC	R--CGAATACGATCGAATCGAACCG	R--ACACCAAAATACAATCAAAATCAACCAAA	
<i>RAR-β</i>	F--TATGYGAGTTGTTGAGGATTGGGA	F--TGTCGAGAACGCGAGCGATTTC	F--TTGGGATGTTGAGAAATGTGAGTGATT	(26)
	R--AATAATCATTTACCATTTTCCAAACTTA	R--CGACCAATCCCAACCGAAACGA	R--CTTACTCAACCAATCCCAACCAAAACAA	

¹We initially used the published primer set to amplify this gene without success. We therefore designed primer sets with MethyI Primer Express Software v 1.0, which did amplify the genes.

Table 2

A. Percent Methylated DNA in Malignant (CA) and Normal (NL) [/] Breast Tissues						
Gene	CA		NL			P Value
	Mean	Median	Mean	Median		
<i>CCND-2</i>	8.66	1.91	0.23	0.15		<0.0001
<i>p16</i>	2.78	0.34	0.39	0.15		<0.0001
<i>RAR-β</i>	6.85	0.32	0.22	0.14		<0.0001
<i>RASSF-1a</i>	45.23	48.58	2.30	1.97		<0.0001
Sample size	44		34			
B. Number (%) Cases Where Fraction Methylated DNA in CA Tissues that Exceeds NL [/] Breast Tissues						
Gene	CA Exceeds 90% of NL		CA Exceeds 100% of NL			
<i>CCND-2</i>	29/44(66)		23/44(52)			
<i>p16</i>	37/44(84)		5/44(11)			
<i>RAR-β</i>	20/44(45)		8/44(18)			
<i>RASSF-1a</i>	35/44(80)		33/44(75)			

[/] reduction mammoplasty samples

Table 3
Percent Methylated DNA in Matched Cancer Tissue (CA), Adjacent Normal Tissue (A), and Nipple Aspirate Fluid (NAF) Samples

A. % Methylation in CA vs. A and NAF									
Gene	CA		A		CA vs. A		NAF		CA vs. NAF
	Mean	Median	Mean	Median	P Value		Mean	Median	P value
<i>CCND-2</i>	6.32	1.18	0.41	0.08	0.002		1.49	0.001	0.01
<i>p16</i>	0.19	0.04	0.007	0.002	0.0001		0.22	0.02	0.12
<i>RAR-β</i>	35.92	17.29	6.77	3.61	0.004		26.14	11.69	0.44
<i>RASSF-1a</i>	63.70	83.96	11.19	1.24	0.0001		16.25	1.21	0.001
Sample size	18		18						
B. % Methylation in NAF vs. A									
Gene	NAF		A		NAF vs. A				
	Mean	Median	Mean	Median	P value				
<i>CCND-2</i>	1.49	0.001	0.41	0.08	0.18				
<i>p16</i>	0.22	0.02	0.007	0.002	0.0001				
<i>RAR-β</i>	26.14	11.69	6.77	3.61	0.067				
<i>RASSF-1a</i>	16.25	1.21	11.19	1.24	0.93				
Sample size	18		18						

Table 4
Percent Methylated DNA in Ductoscopic Samples Collected from Cancer Containing (CA), Precancer Containing (P) and Benign (B) Breasts

A. Cancer (CA) vs. Precancer (P) vs. Benign (B) [/]				
Gene	CA	P	B	P Values
	Median	Median	Median	(C-P-B)
<i>CCND-2</i>	0.02	0.007	0.013	0.24
<i>RAR-β</i>	0.02	0.014	0.027	0.52
<i>RASSF-1a</i>	0.90	0.42	0.32	0.001
<i>p16</i>	0.18	0.17	0.054	0.032
Sample size	61	10	48	0.12
				0.013
B. Number (%) of Cases Where Fraction Methylated DNA in Cancer Exceeds Benign MD Samples				
Gene	CA Exceeds 90% of B	CA Exceeds 100% of B		
<i>CCND-2</i>	18/61(30)	16/61(26)		
<i>p16</i>	11/61(18)	2/61(3)		
<i>RAR-β</i>	9/61(15)	7/61(11)		
<i>RASSF-1a</i>	21/61(34)	11/61(18)		

[/] Cancer includes ductal carcinoma *in situ* and invasive disease; precancer includes atypical hyperplasia and lobular carcinoma *in situ*; benign includes all diagnoses that are not either cancer or precancer.