Array-Based Multiplex Analysis of DNA Methylation in Breast Cancer Tissues

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Abnormal DNA methylation is well established for cancer cells, but a methylation-based diagnostic test is yet to be developed. One of the problems is insufficient accuracy of cancer detection in heterogeneous clinical specimens when only a single gene is analyzed. A new technique was developed to produce a multigene methylation signature in each sample, and its potential for selection of informative genes was tested using DNA from formalin-fixed, paraffin-embedded breast cancer tissues. Fifty-six promoters were analyzed in each of 138 clinical specimens by a microarray-based modification of the previously developed technique. Specific methylation signatures were identified for atypical ductal hyperplasia, ductal carcinoma *in situ*, and invasive ductal carcinoma. Informative promoters selected by Fisher's exact test were used for composite biomarker design using naïve Bayes algorithm. All informative promoters were unmethylated in disease compared with normal tissue. Cross-validation showed 72.4% sensitivity and 74.7% specificity for detection of ductal carcinoma *in situ* and invasive ductal carcinoma, and 87.5% sensitivity and 95% specificity for detection of atypical ductal hyperplasia. These results indicate that informative cancer-specific methylation signatures can be detected in heterogeneous tissue specimens, suggesting that a diagnostic assay can then be developed. (J Mol Diagn 2008, 10:93–101; DOI: 10.2353/jmoldx.2008.070077)

Early detection of breast cancer improves survival rates and quality of life, so screening for breast cancer is an important target of public health.¹ Screening by mammography affords early detection, but its sensitivity is influenced by many factors, including tissue density and the stage of the disease.²

DNA methylation is an attractive paradigm for cancer detection in that differential methylation of multiple genes in normal versus tumor tissue is well-established.^{3–5} Iden-

tical modification of DNA in multiple sites allows testing of multiple biomarker candidates by the same technique. Although analysis of each separate biomarker may not be adequate for diagnosis, combinations of biomarkers can produce accurate assays for cancer detection. Such assays together with the presence of abnormally methylated DNA in the blood of cancer patients^{6,7} create a possibility for a minimally invasive diagnostic test.

We have developed a platform for multiplex detection of DNA methylation at multiple genomic sites⁸ and tested its performance in DNA from fixed human tissues.⁹ Here, we present proof-of-principle data on selection of informative methylated or unmethylated promoter sequences for cancer detection using DNA from gross sections of formalin-fixed, paraffin-embedded clinical specimens. Our approach allows detection of pathological changes via an observer-independent assay, which has obvious advantages for clinical practice.

Materials and Methods

Clinical Samples

The project was approved by the Institutional Review Board of Northwestern University. All samples were collected using Institutional Review Board-approved protocols, evaluated by a pathologist, and stored as formalin-fixed, paraffin-embedded blocks. They were identified by Surgical Pathology Final Reports (without personal data), and reviewed by one of the authors (E.L.W.). One 10- μ m section was used for DNA isolation. There were no attempts to isolate tumor cells or to remove uninvolved areas. The ethnicity of the subjects was not considered. The ages of the subjects and tumor characteristics are presented in Table 1.

Infiltrating ductal carcinoma (IDC) was defined as malignant mammary epithelial cells invading stroma. Samples of well, moderately, and poorly differentiated IDC were examined. Most samples were invasive carcinoma with accompanying ductal carcinoma *in situ* (DCIS). DCIS was defined as malignant mammary epithelial cells con-

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Table 1. Characteristics of Clinical Specimens

	Tissue type			
	DCIS (n = 28)	IDC (n = 39)	ADH (n = 40)	Normal (n = 31)
Age				
Mean (SD)	58.8 (11.1)	52.2 (13.3)	57.6 (11.6)	33.2 (10.5)
Range	40 to 81	33 to 80	36 to 91	22 to 61
P value*		<0.001		
Grade				
1	10	2	ND	ND
2	9	5	ND	ND
3	9	32	ND	ND
P value†		<0.001		
Estrogen receptor				
Fraction positive	1	0.55	NA	NA
Reported value	0.64 ¹³	0.64 ¹³	NA	NA
P value‡	<0.001	0.31		
Progesterone receptor				
Fraction positive	0.75	0.5	NA	NA
Reported value	0.57 ¹³	0.57 ¹³	NA	NA
P value‡	0.06	0.42		
TP53				
Fraction positive	0.19	0.47	NA	NA
Reported value	0.185 ¹⁴	0.53 ¹⁵	NA	NA
P value‡	0.81	0.51		

*P value from ANOVA model of age on tissue type; Bonferroni corrected P values for pairwise comparisons demonstrate a significant difference in the normal group compared with all others ($P < 0.001$).

†P value from Fisher's exact test analog for 3×2 table comparing DCIS and IDC grades.

‡P value from exact binomial test comparing observed proportions to literature-reported values.

tained within ducts or duct-like structures. Samples contained well, moderately, and poorly differentiated DCIS, whereas samples with invasive carcinoma were excluded. Atypical ductal hyperplasia (ADH) was defined according to Page and colleagues¹⁰ and MacGrogan and Tavassoli¹¹ as lesions having all of the characteristics of low-grade DCIS but less than 2 mm in size or, if larger lesions, having only some characteristics of DCIS. Samples with papillomas and radial scars with atypical hyperplasia were sometimes present, but those with DCIS and/or IDC or more advanced disease were excluded. Normal breast tissue samples from reduction mammoplasty (diagnosis of macromastia) either contained no pathological changes or the changes were minimal (fibrosis and fibroadenoma).

DNA Isolation

After xylene deparaffination and ethanol precipitation, the tissue pellet was processed using a DNeasy Tissue kit (Qiagen, Valencia, CA). Purified DNA was dissolved in 10 mmol/L Tris, pH 7.8, 0.5 mmol/L EDTA.

Microarray-Mediated Methylation Assay: Overall Approach

In the microarray-mediated methylation assay (M^3 -assay), one portion of each genomic DNA sample was digested with a methylation-sensitive restriction enzyme, whereas another portion of the same sample served as an undigested control. Selected regions of the genomic DNA from each of the digested and undigested DNA samples were amplified by PCR using gene-specific primers that flank restriction sites. For the amplified prod-

uct from the digested portion, only fragments with methylated sites were capable to serve as templates, whereas in the undigested (control) portion, all fragments were amplified. Comparison between the two sets of PCR products was done by gel electrophoresis (MSRE-PCR)⁸ or by competitive hybridization with custom-designed microarrays (M^3 -assay). Fluorescent signals of hybridized fragments in the M^3 -assay were separately scored, and the ratio between the signals from control and digested DNAs was calculated. This ratio was used to assign "methylated" or "unmethylated" calls to the targeted regions. The data were statistically assessed to select groups of informative fragments, which were then analyzed together as a composite biomarker. Details of the method are presented below.

Microarray-Mediated Methylation Assay

DNA Digestion

Hin6I (Fermentas, Hanover, MD) was used to digest one-half of each purified genomic DNA sample as described previously.⁸ The second half of each DNA sample was incubated in the digestion buffer without the enzyme and served as the control.

PCR Amplification

Nested PCR was performed as described previously.⁸ KlenTaq1¹² (DNA Polymerase Technology, St. Louis, MO) was used at 8 U per 30- μ l reaction. Betain and dNTPs (Sigma, St. Louis, MO) were added to the PCR buffer to 1.5 mol/L and 0.25 mmol/L, respectively. The PCR reaction was assembled on ice, the tubes were

placed into a thermocycler (ABI 9600; Applied Biosystems, Foster City, CA) and incubated at 95°C for 5 minutes, and KlenTaq1 was added. After 25 cycles (95°C for 45 seconds; 62°C for 1 minute; 72°C for 1 minute), the products were precipitated and dissolved in 10 mmol/L Tris, pH 7.8, 0.5 mmol/L EDTA, and 1.5 ng was used for the second PCR, assembled with aminoallyl-dUTP (Biotium, Hayward, CA) and dTTP (3:1), and performed as the first. PCR products were precipitated and dissolved for labeling in 20 μ l of 100 mmol/L NaHCO₃ buffer (pH 9.0).

DNA Labeling

Five microliters of Cy3 or Cy5 (Monoreactive Dye Pack, Amersham, Piscataway, NJ) in dimethyl sulfoxide were dried in a vacuum, and PCR products were added for 2 hours at room temperature. Unreacted dyes were quenched by 10 μ l of 4 mol/L hydroxylamine, and the products were precipitated. The PCR products from undigested (control) DNA were labeled with Cy5, whereas Cy3 was used to label the PCR products from *Hin*6I-digested DNA.

Hybridization and Signal Detection

Custom-designed arrays (MWG Bioinformatics, High Point, NC) containing 60-mer probes for each amplified product were printed in triplicate on aminosilane-modified glass by Microarrays, Inc. (Nashville, TN). The slides were prehybridized for 1 hour at 42°C in 5 $\times$ SSC, 0.1% SDS, and 1% BSA; rinsed with deionized water; and dried. Labeled DNA was dissolved in the hybridization buffer (100 μ l; Ocimum Biosolutions, Indianapolis, IN), denatured (2 minutes at 95°C), and quenched on ice. Microarray GeneFrames (AbGene, Rochester, NY) were used to create space between the slide and the coverslip. Denatured DNA was added, the coverslip was sealed, and the slides were incubated 18 hours at 42°C. The GeneFrame and the coverslip were removed, and the slides were washed at 42°C for 5 minutes in 1 $\times$ SSC, 0.1% SDS, and twice for 5 minutes in 0.1 $\times$ SSC and 0.1% SDS. Slides were scanned using ScanArray XL4000 (sensitivity, ≤ 0.1 molecule/ μ m²; Perkin Elmer, Boston, MA) with ScanArray software. Intensity of each fluorophor was measured for each spot, and the background values were subtracted. Ratios of Cy5-to-Cy3 fluorescence were calculated to compare the yields of PCR products from control and *Hin*6I-digested DNA.

Statistical Analysis

Methylation calls were made independently for each spot, and final gene-specific calls were made according to the majority call from the triplicate spots for that gene. Nonspecific filtering removed uninformative spots; informative genes were selected by Fisher's exact test for differential methylation in each pairwise analysis. Naïve Bayes classification with uninformative prior was used to classify samples assuming that methylation was indepen-

dent for each of the analyzed sites. The predictive ability of the naïve Bayes classifier for all four pairwise comparisons (cancer versus normal, IDC versus normal, DCIS versus normal, and ADH versus normal) was evaluated using fivefold cross-validation. The data were partitioned into five sets with equal distribution of each type of specimens. Each set then served as a test set based on training of the naïve Bayes classifier with the other four sets. The number of misclassifications was counted over all five runs and over 25 random partitions of the data into five groups. Gene selection and classifier parameter estimation were performed anew with each round of cross-validation.

Assessment of Assay Variability

Methylation profiling of genomic DNA of MCF-7 was repeated five times. Forty-nine spots were unambiguously detected, and their methylation calls were independently established for each experiment, creating 49 groups (the number of fragments) of five calls each (five repeats). All calls different from the majority were counted; the number of these calls divided by the total number of calls was used as a measure of the assay's variability.

Results

In this project, we evaluated the possibility of observer-independent analysis of heterogeneous clinical samples with the overall goal of identifying DNA fragments informative for cancer detection. DNA methylation signatures were created for each sample using the microarray-mediated methylation assay (M³-assay) developed in our laboratory (Figure 1). Formalin-fixed paraffin-embedded breast tissues were used.

Clinical Samples

The most advanced stage in each sample was used to assign samples to ADH, DCIS, and IDC groups, so tumors with IDC could contain regions with DCIS and ADH, whereas DCIS samples could include regions with ADH. To ensure observer-independent evaluation, we did not microdissect tumor-containing regions.

Age distribution was similar within each group (Table 1). The mean age was lower for reduction mammoplasty (normal) group ($P < 0.001$ using an analysis of variance model). The age difference was significant between the normal and other groups (adjusted P values < 0.001 in pairwise comparisons with Bonferroni adjusted P values). Data on the expression of estrogen and progesterone receptors and p53 were not available for ADH and normal samples. In DCIS, the fraction of estrogen receptor-positive tumors (100%) was higher than reported ($P < 0.001$), but the fraction of progesterone-positive tumors (75%) was similar.¹³ In IDC, the fraction of tumors expressing estrogen and progesterone receptors was consistent with reported values.¹³ The percentage of p53-

Figure 1. The M^3 assay. General schema of the assay: Isolated DNA is divided into two aliquots; one of them is incubated with *Hin6I*, and the other is left untreated. Both are used for PCR amplification with gene-specific primers; the products are labeled with different fluorophores, mixed, and used for competitive hybridization with the array. After signal processing and statistical analysis, selected diagnostic gene set is evaluated in all specimens.

positive tumors was close to reported for both DCIS¹⁴ and IDC¹⁵ groups.

M^3 -Assay

DNA methylation analysis was performed as shown in Figure 1. Fifty-six promoter fragments were interrogated (Figure 2) in each experiment. Negative control fragments included coding sequences of three genes (asterisk in Figure 2) and heterologous DNA from *Arabidopsis thaliana*. Each probe on the array was designed to detect corresponding PCR product. Each microarray contained

ABCB1	ACTB	ACTB*	APAF1	Arabidops.	BRCA1	CALCA	CASP8
CCND2	CDH1	CDKN1A	CDKN1B	CDKN1C	CDKN2A	CDKN2B	DAPK1
DNAJC15	EDNRB	EP300	ESR1prA	ESR1prB	FABP3	FAS	FHIT
GAPDH*	GPC3	GSTP	HIC1	HLTF	ICAM1	MCTS1	MGMT
MLH1	MSH2	MUC2	MYOD1	NR3C1	PAX5	PGK1	PGRdist
PGRprox	PLAU	PRDM2	PRKDCBP	PYCARD	RARB	RASSF1	RB1
RPL15	S100A2	SCGB3A1	SFN	SLC19A1	SOC51	SYK	TES
THBS1	TNFSF11	TP73	TUBA3*	VHL			

Figure 2. Genes present on the microarray. The microarray contains 64 positions (8×8 format) with 3 empty and 61 occupied spots. Three spots (*ACTB**, *GAPDH**, and *TUBA3**) contain probes for transcribed sequences of corresponding genes, and another spot is occupied by a probe for genomic DNA of *A. thaliana*. One of the remaining probes (*HLTF*) is defective. Accordingly, 61 occupied spots contain 4 controls and 1 defective probe, leaving 56 spots for analysis. Two promoters are evaluated for *ESR1* (A and B) and *PGR* (proximal and distal).

Table 2. SMC-Based Call Assignment*

Gene	Cy5	Cy3	Ratio	Methylation call
<i>MDR1</i>	64,400	64,946	1.0	M
<i>SFN</i>	64,450	64,976	1.0	M
<i>CDKN2B</i>	64,547	63,763	1.0	M
<i>RPL15</i>	64,524	60,570	1.1	M
<i>PGK1</i>	64,510	50,217	1.3	M
<i>FABP3</i>	64,490	40,435	1.6	M
<i>RASSF1</i>	10,212	6360	1.6	M
<i>BRCA1</i>	64,504	36,053	1.8	M
<i>PAX5</i>	64,561	33,619	1.9	M
<i>DNAJC15</i>	64,504	32,923	2.0	M
<i>SLC19A1</i>	17,732	8786	2.0	M
<i>EDNRB</i>	44,391	17,758	2.5	M
<i>ESR1</i> promoter A	5807	2210	2.6	M
<i>CDKN1C</i>	37,616	13,193	2.9	M
<i>MCTS1</i>	64,509	17,836	3.6	M
<i>TNFSF11</i>	15,402	1389	11.1	U
<i>CDH1</i>	6044	508	11.9	U
<i>ICAM1</i>	51,208	3997	12.8	U
<i>EP300</i>	64,551	4781	13.5	U
<i>PGR</i> distal promoter	61,207	2653	23.1	U
<i>TP73</i>	31,236	1304	24.0	U
<i>MGMT</i>	64,423	2336	27.6	U
<i>MSH2</i>	50,032	534	93.8	U

M, methylated; U, unmethylated.

*SMC = 4.0.

three identical subarrays, so that every hybridization signal was confirmed in triplicate. Unreliable hybridization signals with intensities comparable with or less than background were excluded, and background was subtracted. The threshold for methylation was determined experimentally using “self-self” hybridizations¹⁶; ie, PCR products from control (undigested) DNA were divided into two equal aliquots, labeled with either Cy3 or Cy5, mixed, and hybridized to the array; the average Cy5-to-Cy3 ratio was recorded. This “self-self” design assured equal representation of Cy3- and Cy5-labeled fragments as would be expected from samples of methylated DNA. This average ratio of intensities was used as a threshold to define methylation [standard methylation call (SMC)]. SMCs were used to assign calls for each gene: methylated to genes with $Cy5/Cy3 \leq SMC$ and unmethylated to genes with $Cy5/Cy3 > SMC$. An example of data are shown in Table 2. If no call could be assigned, the gene was scored as nonapplicable.

Validation of the Assay

A previously validated procedure (MSRE-PCR)⁸ was used for methylation detection. Every assay included two stages: 1) detection of methylation by MSRE digestion, and 2) detection of the signal for each promoter fragment. Briefly, the analytical sensitivity of the assay was determined to be 60 pg for one gene in MSRE-PCR⁹ or 100 pg for multiple genes in M^3 -assay (data not shown). Digestion was confirmed by real-time PCR for selected genes,⁸ by detection of unmethylated genes in the M^3 -assay, and by preservation of methylation patterns in experiments with increased digestion (data not shown). Similar, if not identical, methylation patterns were de-

Figure 3. Performance of the M^3 -assay with heterogeneous samples. Genomic DNA from MCF7 and T47D was mixed at a different ratio and used for analysis. Methylation status of *MYF3*, *PAX5*, *RPL15*, and *RB1* was determined as described and plotted against the percentage of unmethylated genes. Cy5-to-Cy3 ratio remains at the level of SMC for all genes with no less than 50% of methylated fragments, and such genes are scored as methylated. Further increase in Cy5-to-Cy3 ratio reflects prevalence of unmethylated fragments in the sample.

tected by the MSRE-PCR and bisulfite-based assays (methylation-sensitive PCR and bisulfite sequencing⁸); in addition, comparison of MSRE-PCR data with published results revealed a remarkable degree of correlation.⁸

No attempt was made to correlate the results of the M^3 -assay and expression profile of analyzed samples. By its design, the M^3 -assay assessed methylation only in a few CpG sites in each promoter, so a rigorous correlation between gene expression and methylation results could not be expected.

Reproducibility of the M^3 -assay was evaluated using genomic DNA from MCF-7 cells. The assay was repeated five times, and the readout was evaluated for each fragment as described in Materials and Methods. Six of 245 total data points were variable (2.4%), suggesting a variability of less than 3% for the assay.

We also evaluated the link between the Cy5-to-Cy3 ratio and the level of methylation in heterogeneous samples. Control samples were prepared using a mixture of genomic DNA from MCF-7 and TD47D cells so that each sample contained a predetermined percentage of methylated and unmethylated genes. Cy5-to-Cy3 ratios below SMC were observed for samples with up to 50% unmethylated DNA (Figure 3). Samples with greater than 50% unmethylated genomic DNA fragments caused gradual increases of the Cy5-to-Cy3 ratio (Figure 3). These results indicate that the efficient detection of methylated fragments incorporated in the MSRE-PCR procedure⁸ was preserved in the M^3 -assay.

The likelihood of potential PCR bias in the M^3 -assay was reduced by the use of the same sets of primers and amplification conditions for digested and control DNA, so controllable parameters (DNA concentration, amplicon length, primer concentration, etc) were identical. Each specimen contained multiple genes that produced high signal in digested sample and were scored as "methylated" based on the selected criteria, thus providing direct evidence against such a bias. Each sample also contained several genes that were scored as "unmethylated", thus providing evidence that *Hin6I* digestion was efficient.

Classification of Samples

Each subarray contained 61 fragments and three empty spots (Figure 2) producing 192 spots on the array, 183 of which contained probes. Methylation calls were made in a blinded manner and independently for each spot. The majority call for the three spots for each gene was assigned as a final gene-specific methylation call. If there was no majority, the final call was nonapplicable. In a total of 8418 calls made for 61 genes in each of 138 samples, 4725 were methylated (56.1%), 2045 were unmethylated (24.3%), and 1648 (19.6%) were nonapplicable.

Similar to expression microarray analysis,¹⁷ nonspecific filtering was used to eliminate uninformative genes with detectable calls in less than two-thirds of the samples or less than 10% differential methylation across the entire sample set (eg, 90% methylated and 10% unmethylated). Nonspecific filtering steps were repeated for four pairwise analyses, but only a few genes were eliminated, and more than 45 genes were selected for each comparison: DCIS versus normal, 46 genes; IDC versus normal, 48 genes; DCIS/IDC versus normal, 48 genes; and ADH versus normal, 49 genes. Informative features for classifiers were selected with Fisher's exact test using $P < 0.10$. The moderate P value of 0.10 was chosen to narrow the set of genes but to include informative genes with occasionally inflated P values.

The apparent independence of methylation sites¹⁸ suggested selection of the naïve Bayes classifier,¹⁹ which performed surprisingly well even when independence was not satisfied.²⁰ Naïve Bayes classifiers were constructed using the e1071 R (R Development Core Team, 2005) package,²¹ using an uninformative prior with probabilities of 0.5 for each group in the pairwise classification schemes.

Sensitivity and specificity of the assay and overall classification accuracy were determined (Table 3). Besides DCIS and IDC groups, a combined Cancer group was created, which contained both DCIS and IDC samples.

Classifier Genes

Nine promoters were consistently predictive for cancer classification in all rounds of cross-validation, whereas 19 were important for ADH classification (Table 4). In all cases unmethylated genes were informative; this was consistent with the design of the assay in which a methylated signal would be found even when only a fraction of specific templates was methylated.⁸ In this respect, the M^3 -assay performed very similar to the original MSRE-PCR assay (Figure 3). In a heterogeneous specimen, a methylated sequence could originate from tumor cells or any other part of the sample, would nonetheless be amplified, and the whole fragment would be scored as methylated. Only unmethylated fragments could be unequivocally assigned to tumor cells, and their unmethylated status in other parts of the sample would not change the result of the M^3 -assay.

Table 3. Performance of M³-Assay

		True status		
		Cancer	Normal	
Predicted status	pCancer	0.7239	0.2526	1. Cancer classifier
	pNormal	0.2761	0.7474	
		ADH	Normal	
	pADH	0.8750	0.0501	2. ADH classifier
	pNormal	0.1250	0.9499	
		DCIS	Normal	
	pDCIS	0.7048	0.1869	3. DCIS classifier
	pNormal	0.2952	0.8131	
		IDC	Normal	
	pIDC	0.7056	0.2686	4. IDC classifier
	pNormal	0.2944	0.7314	

Cancer is any sample with either DCIS or IDC component. Predicted status for each sample (eg, pCancer, pADH, pNormal, etc.) was compared with its true status (cancer, ADH, normal, etc.). Intersection of predicted and true status for each type of cancer shows the sensitivity (eg, 72.39% of cancer samples are correctly identified, so the sensitivity of cancer classifier is 72.39%), whereas intersection of predicted and true status of normal samples indicates the specificity of the classifier (eg, 74.74% of normal samples are correctly identified by the cancer classifier, so its specificity is 74.74%).

Discussion

Technical Approach

Abnormal DNA methylation in neoplastic cells can be a valuable biomarker for cancer detection.^{22,23} Unfortunately there is only a limited probability of methylation for each gene,²⁴ so only a combined measurement of multiple methylation biomarkers may provide useful data. The M³-assay is developed to generate such composite biomarkers.

Use of bisulfite degrades the target DNA (up to 95%)²⁵ and hence may reduce amplifiable DNA.²⁶ Biased amplification of remaining DNA (sequence-, strand-, and level of methylation-dependent bias) has been reported.²⁷ Although these problems may not be significant for

homogeneous or ample specimens, they can be critical for heterogeneous clinical specimens and may produce inaccurate results, especially if DNA degradation is specific to certain sequences. In addition, degradation of the major part of a limited clinical sample may prevent its comprehensive analysis, which will be also reflected in reduced analytical sensitivity. With this in mind, we have compared bisulfite-based techniques (methylation-specific PCR and bisulfite sequencing) with MSRE-PCR using homogeneous specimens from cultured cells where these problems are less likely to produce biased results.⁸ The inherent flaws in the bisulfite technique suggest that an alternative procedure for detection of methylated DNA in clinical samples is needed.

Table 4. Genes Used for Classifier of Each Sample Group

	Fraction unmethylated (Fisher's exact test <i>P</i> values for pairwise comparison with Normal group)				
	Normal	ADH	DCIS	IDC	Cancer*
<i>EP300</i>	0.167	0.675 (<0.001)	0.577 (0.002)	0.474 (0.010)	0.516 (0.001)
<i>MGMT</i>	0.379	0.925 (<0.001)	0.852 (<0.001)	0.744 (0.003)	0.788 (<0.001)
<i>TP73</i>	0.103	0.750 (<0.001)	0.520 (0.001)		0.410 (0.003)
<i>PGR</i> (distal pr)	0.346	0.842 (<0.001)		0.657 (0.021)	0.639 (0.018)
<i>THBS1</i>	0.233	0.750 (<0.001)		0.526 (0.024)	0.515 (0.014)
<i>PYCARD</i> (<i>TMS1</i>)	0.200	0.889 (<0.001)	0.545 (0.018)	0.706 (<0.001)	0.643 (<0.001)
<i>PRKDCBP</i> (<i>SRBC</i>)	0.269	0.826 (<0.001)	0.647 (0.026)		
<i>FABP3</i> (<i>MDGI</i>)	0.333			0.724 (0.009)	0.660 (0.018)
<i>MSH2</i>	0.385		0.875 (<0.001)		0.750 (0.003)
<i>HIC1</i>	0.100		0.444 (0.006)	0.395 (0.011)	0.415 (0.002)
<i>BRCA1</i>	0.032	0.650 (<0.001)			
<i>TES</i>	0.000	0.600 (<0.001)			
<i>NR3C1</i> (<i>GR</i>)	0.032	0.550 (<0.001)			
<i>ICAM1</i>	0.214	0.781 (<0.001)			
<i>DAPK1</i>	0.161	0.600 (<0.001)			
<i>TNFSF11</i> (<i>RANKL</i>)	0.194	0.641 (<0.001)			
<i>DNAJC15</i> (<i>MCJ</i>)	0.346	0.800 (<0.001)			
<i>CDH1</i>	0.308	0.760 (0.002)			
<i>CASP8</i>	0.269	0.641 (0.005)			
<i>RPL15</i>	0.231	0.550 (0.012)			
<i>PGK1</i>	0.179	0.475 (0.019)			

The fraction of unmethylated calls for each tissue type is shown with *P* values from Fisher's exact test for differential methylation on 2 × 2 tables for all pairwise comparisons. These values are reported only as summary statistics. In the cross-validation scheme, gene selection was performed separately for each training set (see text). Blank cells indicate that the gene was not consistently selected in the classifier for the corresponding comparison.

*Cancer is any sample with either DCIS or IDC component.

The M³-assay is similar to MSRE-PCR⁸ but relies on microarray-based rather than gel-based signal detection. As in many other DNA methylation techniques, the M³-assay evaluates methylation in a selected number of sites in each gene that may or may not correlate with sites critical for gene expression; this feature makes direct comparison of methylation and expression tenuous.⁸ The M³-assay is designed to efficiently detect methylated DNA fragments that can serve as templates for PCR in a heterogeneous sample. In the heterogeneous sample, any component can provide such a fragment, making it impossible to explicitly assign methylation to a specific part of the sample, eg, to neoplastic cells. The absence of PCR product, on the other hand, indicates that no tissue within the sample contains methylated fragments, so the absence of methylation in neoplastic tissue can be unequivocally established. This feature of the M³-assay makes the detection of unmethylated genes informative for specimen classification, whereas detection of methylated genes is uninformative.

Assignment of “methylated” and “unmethylated” calls in the M³-assay depends on the ratio of fluorescence produced by undigested and digested DNA, which in theory can only assume two values: $1/1 = 1$, if the fragment is methylated and digestion has no effect, or $1/0 = \text{infinity}$, if the fragment is unmethylated and no signal from digested DNA is detected. This type of ideal distribution is rarely seen even in cell lines.⁸

Quantitative measurement of signals expressed as Cy5-to-Cy3 ratio can produce significant discrepancies due to variability of experimental conditions and sampling differences. To manage experimental variability (eg, the dye bias), SMC is used to define a threshold for methylation (a “self-self” hybridization¹⁶). This approach reduces numerical microarray data to a binary readout (Table 2), simplifies downstream analysis, and reduces the influence of sampling errors. As with the MSRE-PCR, the M³-assay efficiently detects methylated genes. For example, a sample containing equal amounts of methylated and unmethylated fragments (50% unmethylated) produces a “methylated” readout (Figure 3). Further increase of the share of the unmethylated fragment drives the Cy5-to-Cy3 ratio above the SMC level, so these fragments are scored as “unmethylated.” Interestingly, the increase in the Cy5-to-Cy3 ratio is different for analyzed genes, suggesting certain influence of nucleotide composition on dye incorporation; for *PAX5* even 10% of methylated fragments keep the Cy5-to-Cy3 ratio rather low (Figure 3).

Importantly, the M³-assay is not intended for quantitative assessment of methylation: it is designed for analysis of heterogeneous clinical samples where quantitative differences in methylation can depend on many reasons, including variations in tumor-to-stroma ratio and presence or absence of inflammation. These variations can be reduced by careful selection of samples but at the cost of their subjective evaluation.

Another feature of the M³-assay is the internal control for each spot provided by undigested DNA. This control is essential when damaged DNA (eg, DNA from formalin-fixed, paraffin-embedded samples) is used to ensure that

a specific fragment is present. Data processing ignores all spots where hybridization signals for control (undigested) DNA are not detected.

Because of technical challenges of microarray-based techniques, the M³-assay is not intended for immediate clinical use; rather, the M³-assay provides the screening tool for selection of informative genes for a specific disease. Once such genes are identified, other, less demanding techniques can be applied to design the final clinical test.

Classifier Genes

The Classifier for Cancer is a combination of DCIS and IDC classifiers (Table 4). For example, *TP73* and *MSH2* are components of the DCIS but not of the IDC classifier, indicating differences important only to ductal carcinoma *in situ*. Conversely, *PGR*, *THBS1*, and *FABP3* are not informative for DCIS classification but contribute to IDC classification, suggesting that disparities in their methylation status are significant only in invasive cancer.

Most of the promoters that define the Cancer classifier (six of nine) are also components of the ADH classifier, a result consistent with previously reported data that cancer-defining methylation changes appear very early in the process,²⁸ extending these findings to unmethylated genes. Presence of *PRKCDP* within the ADH and DCIS classifiers may indicate methylation changes that are informative during early stages of breast cancer but not during IDC. Unmethylation calls for each gene and *P* values from Fisher's exact test for all pairwise comparisons are shown (Table 4). Blank cells indicate that the gene was not selected for the biomarker.

It is important that a useful biomarker for cancer contains unmethylated rather than methylated genes, because in a heterogeneous tissue, a methylated fragment may be amplified from any part of the sample, so the methylation signal is not necessarily produced by the tumor. Absence of methylation, however, explicitly indicates that the fragment is unmethylated everywhere in the sample, including tumor cells, so the difference in unmethylated genes between healthy tissue and cancer specimen can be used to identify tumors. It is expected that genes that are unmethylated in tumor but methylated in healthy tissue can be related to tumor growth, dedifferentiation, and invasiveness. At least some of the genes found in our study meet these criteria (eg, *EP300*,²⁹ *TP73*,³⁰ *THBS1*,³¹ and *FABP3*³²).

The larger number of informative promoters identified for the ADH classifier (Table 4) is reflected in a higher accuracy of the ADH classifier (Table 3), suggesting a systematic difference. The most consistent difference is the source of specimens in that all samples of ADH are from core biopsies, whereas other specimens are from gross sections of surgically removed tissues. These gross sections have not been enriched for tumor cells and contain variable amounts of stroma and tumor cells. Compared with gross sections, core biopsies of ADH are by far the most homogeneous.

The similarities in sets of informative genes found for the different stages of breast cancer indicate that no

substantial difference can be detected and that differentiation of these stages is currently impossible. These observations raise two distinct possibilities, either that the current set of genes is insufficient to define specific biomarkers for each stage or that progression of breast cancer from ADH to IDC does not involve molecular differences, at least at the level of DNA methylation. Although there is no data to test either hypothesis, we believe that inclusion of additional genes will create a larger analytical space and will provide new biomarkers specific for each stage of breast cancer.

Results of this study may be affected by the age difference in the control and other groups (Table 1) because DNA methylation increases with age.³³ However, informative genes are chosen for their reduced methylation in abnormal samples, so it is unlikely that age-dependent increase of methylation has significantly influenced the results.

Although abnormal promoter methylation is an established feature of breast cancer cells,³⁴ a diagnostic test based on DNA methylation has yet to be developed. One of the problems is the variability of methylation for each individual fragment. This variability indicates that analysis of a single gene may not provide sufficient accuracy for cancer detection. In the last 2 years, several groups reported multigene DNA methylation profiles for detection and classification of breast cancer,^{35–39} so the need for multigene profiles is widely recognized. The M³-assay is designed to quickly generate such profiles, facilitating selection of informative genes that can become targets for a clinical test.

Importantly, the M³-assay produces an integral methylation profile, where the signal from tumor cells is merged with signal from other tissues. As a result, the methylated call can be produced by any or all parts of the sample, so the informative value of the methylated calls is much lower than that of the unmethylated, which indicates that the fragment is unmethylated in all parts of the sample. Low informative value of the methylated calls explains why the composite biomarker contains only the unmethylated calls. This feature complicates direct comparison with data from other studies, where hypermethylation of a specific promoter is informative. Results of Fackler et al³⁹ demonstrate this difference: All hypermethylated (and thus informative) promoters of their study tested in our project, are scored as methylated (and thus uninformative) by the M³-assay.

This study shows that complex and heterogeneous samples can be classified if methylation in multiple sites within the same specimen is evaluated. The current version of the assay is still insufficiently accurate and too complex for clinical application; however, it provides the platform for selection of informative genes that can produce a composite biomarker. Furthermore, tissue analysis has only limited clinical utility and serves only as a proof-of-principle that a combined analysis of multiple informative genes in heterogeneous samples is feasible and may lead to development of an accurate composite biomarker. It is possible that using the same assay with cell-free circulating DNA may provide a useful approach for cancer detection.

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