Technical Advance

Methylation Target Array for Rapid Analysis of CpG Island Hypermethylation in Multiple Tissue Genomes

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Hypermethylation of multiple CpG islands is a common event in cancer. To assess the prognostic values of this epigenetic alteration, we developed Methylation Target Array (MTA), derived from the concept of tissue microarray, for simultaneous analysis of DNA hypermethylation in hundreds of tissue genomes. In MTA, linker-ligated CpG island fragments were digested with methylation-sensitive endonucleases and amplified with flanking primers. A panel of 468 MTA amplicons, which represented the whole repertoire of methylated CpG islands in 93 breast tumors, 20 normal breast tissues, and 4 breast cancer cell lines, were arrayed on nylon membrane for probe hybridization. Positive hybridization signals detected in tumor amplicons, but not in normal amplicons, were indicative of aberrant hypermethylation in tumor samples. This is attributed to aberrant sites that were protected from methylation-sensitive restriction and were amplified by PCR in tumor samples, while the same sites were restricted and could not be amplified in normal samples. Hypermethylation frequencies of the 10 genes tested in breast tumors and cancer cell lines were 60% for *GPC3*, 58% for *RASSF1A*, 32% for *3OST3B*, 30% for *HOXA5*, 28% for *uPA*, 25% for *WT1*, 23% for *BRCA1*, 9% for *DAPK1*, and 0% for *KL*. Furthermore, hypermethylation of 5 to 7 loci of these genes was significantly correlated with hormone receptor status, clinical stages, and ages at diagnosis of the patients analyzed. This novel approach thus provides an additional avenue for assessing clinicopath-

ological consequences of DNA hypermethylation in breast cancer. (Am J Pathol 2003; 163:37–45)

CpG islands are short stretches of guanine cytosine (GC)-rich sequences frequently located in the promoter and the first exon of genes.¹ In cancer cells, aberrant DNA methylation is known to occur within these regulatory regions. This epigenetic event is somatically heritable and increased density of methylated CpG sites within a promoter region can accumulate over time during tumorigenesis.^{2,3} The methylation build-up is associated with the formation of repressive chromatin and the resulting silencing of the associated gene.³ Accumulated evidence indicates that this epigenetic mechanism is responsible for silencing >100 genes for tumor suppression, genomic instability and repair, apoptosis, cell cycle regulation, and signal transduction in many tumor types.⁴ Hypermethylated CpG islands therefore play a causal role in promoting tumor development and are useful molecular markers for cancer diagnosis and prognosis.

To dissect complex epigenetic patterns in cancer cells, we previously developed a microarray-based approach in which thousands of short CpG island tags known as probes were arrayed on nylon membranes or glass slides.^{5–7} DNA targets representing different pools of methylated CpG islands in tumor or control samples were used to hybridize the microarray panel. Hypermethylated CpG islands were identified in tumor samples based on their differential hybridization patterns relative to normal controls. This genome-wide screening tool provides an efficient way to survey DNA hypermethylation in one tumor at a time and has been used to identify many novel methylation-silenced genes.^{5–7} To further assess

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Table 1. Primers for Linker-PCR, MTA Probes, Methylation-Specific PCR, and Reverse-Transcription PCR

	Oligonucleotide (5'→)		Size (bp)	Annealing temp. (°C)
	Sense primer	Antisense primer		
Linker PCR				
L- <i>Mse</i> I/ <i>Bfa</i> I	TAATCCCTCGGA	AGGCAACTGTGCTATCCGAGGGAT		72
L- <i>Nla</i> III	TCCCTCGGATA	AGGCAACTGTGCTATCCGAGGGACATG		72
MTA probe				
<i>GPC3</i>	GCGACTATTTCAGGTTGGC	TACAGCCTGGAGGGCTTTTC	230	60
<i>WT1</i>	TTAGTCAGGATACAAGGAGG	CGAGTAATTTTGCTCCAGGA	256	53
<i>BRCA1</i>	TAGAGGGCAGGCACCTTTATG	AGGAAGTCTCAGCGAGCTCA	204	56
<i>KL</i>	GCTCCATTGTATTGTAGAGG	GTCGTTGATTGTTCTCCTGA	255	53
<i>uPA</i>	TGCCTTTCCCCCTCTGATAG	TGGAGCTGTGAAGTCTTCATC	219	58
<i>RASSF1A</i>	GTCCGTTACTTTCGCCCCAT	CATGAGTGTTTGAGAAGAGGCA	228	60
<i>HOXA5</i>	TCTCCTTGCCGTTCTCCCTC	CAGCGTAGCCCTCCTCATAA	270	60
<i>p16^{INK4}</i>	ATAGACGTGAGCCACCGCAT	ATGGTGGCGCACGTGTGTAA	219	57
<i>3OST3B</i>	CAGGTTTGAGCCTCGGGTTC	ACA AGGTCGGAGGGTCGTCT	256	62
<i>DAPK1</i>	ACATATTTGGGACTCCAGAG	GGCACTGGTAT TAGTGAAGT	212	57
Methylation-specific PCR				
<i>p16-M</i>	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA		
<i>p16-U</i>	TTATTAGAGGGTGGGGTGGATTGT	CCACCTAAAT CAACCTCCAACCA		
<i>3OST3B-M</i>	AAACCGAAAACGACCG CCGCGCG	GTTTCGCGGTGCGTATCGCGTCGC		
<i>3OST3B-U</i>	GGGTGGATTGAGAGTGATTGTTGTGT	AAAATTCAACAATACACCACACCACAA		
Reverse transcription-PCR				
<i>3OST3B</i>	TTTTTCAGTGGGTCTGGGAGC	CGATGAGCTTG GTGTCCTTG		

the utility of candidate CpG islands as epigenetic markers for tumor progression, we reversed the process in this study by arraying multiple methylation targets on solid supports and hybridizing the array one at a time with different CpG island probes. Similar to the concept of tissue microarray,⁸ this approach, called Methylation Target Array (MTA), has the capacity for simultaneous interrogation of CpG island hypermethylation in hundreds of clinical samples. Here we describe its application in determining hypermethylation profiles of 10 genes in multiple breast tumors. These profiles were correlated with the clinicopathological features of the patients.

Materials and Methods

Tissue Samples and Cell Lines

Tumor tissues were obtained from patients undergoing mastectomy before chemotherapy at the Ellis Fischel Cancer Center in compliance with the Institutional Review Board. Clinicopathological features were determined based on the Tumor-Node-Metastasis system.⁹ All breast tumors analyzed in this study were infiltrating ductal carcinomas. Adjacent normal parenchyma 3 to 5 cm away from the tumor area was obtained to serve as a normal breast tissue control. The estrogen receptor (ER)/progesterone receptor (PR) status of clinical samples was determined by either the dextran-coated charcoal assay or the immunoperoxidase technique, as described.¹⁰ Based on our estimate, 5% to 10% of stroma or infiltrating lymphocytes are present in the collected tumor specimens. These were considered as part of the "texture" of breast tumors, which were used throughout this study without further microdissection to procure pure tumor cells. The presence of residual normal cells was not expected to affect the overall interpretation of the methylation data subsequently acquired. Tumor specimens

contaminated with significant amounts of normal cells were not used in the study. Breast cancer cell lines T47-D, ZR-75, Hs578t, and MDA-MB-231 were maintained as described.⁵ Genomic DNA was isolated using the QIAamp Tissue Kit (Qiagen, Valencia, CA).

Construction of Methylation Target Array

To generate methylation targets, genomic DNA (1 μ g per sample) of multiple tissue samples was individually placed in 96-well format microtubes and digested with *Mse*I, *Bfa*I, *Nla*III, or *Tsp509I* (25 U/well; New England Biolabs, Beverly, MA). The digests were then purified and their sticky ends ligated with 0.5 nmol of unphosphorylated primer linkers (Table 1). The ligated DNA was purified and further digested with methylation-sensitive endonucleases, *Bst*UI (25 U/well, 60°C, 16 hours) and *Hpa*II (25 U/well, 37°C, 16 hours), successively. PCR (300 μ l per tube) was performed in the 96-well format microtubes using the ligated DNAs as templates and a linker as primer, and subjected to 20 cycles of amplification. Amplified targets were concentrated and printed (~0.3 μ l per dot, 0.5 μ g/ μ l) as duplicate microdots onto nylon membranes using the MULTI-PRINT replicator (V&P Scientific, San Diego, CA). Spotted DNA was denatured and UV-crosslinked to the matrix.

MTA Hybridization

Primer sequences and PCR conditions used to prepare probes are listed in Table 1. PCR-generated probes were purified, labeled with ³²P-dCTP, and hybridized with MTA filters at high stringent conditions (68 to 72°C) using the Rapid-Hyb Buffer system (Amersham Biosciences, Inc., Piscataway, NJ). Post-hybridization washing was performed as previously described.⁵ The hybridized filters

Table 2. Survey of Internal Methylation-Sensitive Sites in 100 CpG Islands Frequently Hypermethylated in Various Cancers

Gene	Accession no.	<i>MseI</i>		<i>Tsp509I</i>		<i>NlaIII</i>		<i>Bfal</i>		Gene	Accession no.	<i>MseI</i>		<i>Tsp509I</i>		<i>NlaIII</i>		<i>Bfal</i>	
		B	H	B	H	B	H	B	H			B	H	B	H	B	H	B	H
<i>Stratifin</i>	NM.006142	0	0	0	6	0	2	0	6	<i>PRDM2</i>	NM.012231	15	16	17	16	16	15	18	18
<i>Cyclin A1</i>	NM.003914	15	20	—	—	2	8	14	16	<i>uPA*</i>	NM.002658	10	18	1	1	1	2	7	10
<i>IGF2</i>	NM.000612	—	—	16	19	4	10	17	19	<i>TJP2</i>	NM.004817	18	16	8	6	19	17	19	16
<i>IGFBP7</i>	NM.001553	4	6	16	16	5	7	0	1	<i>KAI1</i>	NM.002231	0	5	2	16	0	0	2	13
<i>ABL1</i>	NM.005157	8	3	0	0	5	7	16	12	<i>MGMT</i>	NM.002412	0	1	0	1	0	0	0	0
<i>IRF7</i>	NM.001572	0	0	0	0	5	10	0	4	<i>MLH1</i>	NM.000249	6	5	5	4	7	6	5	2
<i>STK11</i>	NM.000455	—	—	20	35	20	34	19	26	<i>GSTP1</i>	NM.000852	0	0	1	0	8	9	3	3
<i>NEFL</i>	NM.006158	4	0	7	2	2	4	3	2	<i>BRCA1*</i>	NM.007294	1	1	1	1	3	2	3	3
<i>RASSF1A*</i>	NM.007182	16	23	15	7	10	1	1	0	<i>RPA2</i>	NM.002946	1	3	0	1	3	0	3	0
<i>FHIT</i>	NM.002012	3	4	1	1	1	0	2	3	<i>HOXA5*</i>	NM.019102	12	21	6	9	4	4	7	13
<i>SOCS-1</i>	NM.003745	6	12	0	1	5	13	2	6	<i>TERT</i>	NM.003219	1	5	0	1	5	5	6	8
<i>DAPK-1*</i>	NM.004938	1	4	18	32	3	6	11	20	<i>PTGS2</i>	NM.000963	0	3	0	2	0	3	5	6
<i>HSPA1</i>	NM.005345	1	2	1	1	1	1	1	3	<i>SMI2</i>	NM.009586	—	—	2	2	14	15	8	6
<i>CDKN2B</i>	NM.004936	4	7	2	1	6	8	2	1	<i>TIMP3</i>	NM.000362	0	1	0	1	0	1	0	1
<i>P16*</i>	NM.000077	4	4	1	1	3	5	6	7	<i>AR</i>	NM.000044	3	6	1	0	0	0	1	4
<i>WT1*</i>	AL049692	13	28	13	28	13	26	13	22	<i>ESR1</i>	NM.000125	1	1	0	0	2	3	0	0
<i>VHL</i>	NM.000551	1	3	2	1	1	0	2	1	<i>ASC</i>	NM.013258	0	4	0	1	1	1	0	4
<i>Rb1</i>	NM.000321	1	3	6	4	9	5	0	1	<i>LTB4R</i>	NM.000752	0	1	—	—	1	2	1	2
<i>HIC1</i>	NM.006497	—	—	—	—	11	26	—	—	<i>Calcitonin</i>	NM.001741	5	11	3	5	0	3	3	5
<i>FABP3</i>	NM.004102	2	3	2	4	0	1	0	2	<i>EPHA3</i>	NM.005233	0	2	0	2	0	2	0	2
<i>APC</i>	NM.000038	0	2	0	2	0	1	0	2	<i>EPO</i>	NM.000799	0	2	0	2	1	4	8	16
<i>N33</i>	NM.006765	9	13	9	13	9	13	8	10	<i>CDKN1A</i>	NM.078467	0	2	0	1	18	16	11	10
<i>GSN</i>	NM.000177	3	6	11	6	4	3	10	6	<i>TNFRSF6</i>	NM.000043	0	0	6	9	3	3	—	—
<i>CSPG2</i>	NM.004385	4	3	3	1	0	0	1	0	<i>MYOD1</i>	NM.002478	5	6	0	1	10	11	1	4
<i>KLK10</i>	NM.145888	2	5	2	3	1	1	1	1	<i>ICAM1</i>	NM.000201	4	5	10	10	7	17	2	10
<i>DBCCR1</i>	NM.014618	8	15	10	15	7	12	8	12	<i>PRKCDPB</i>	NM.145040	1	1	1	1	2	1	7	3
<i>CAV1</i>	NM.001753	1	1	0	0	3	3	2	1	<i>Testin</i>	NM.015641	5	8	1	0	5	8	1	0
<i>CDKN1B</i>	NM.004064	2	2	3	7	7	9	3	2	<i>LRP-2</i>	NM.004525	—	—	1	4	2	1	6	2
<i>CDKN1C</i>	NM.000076	5	11	0	0	13	15	14	16	<i>TGFBR1</i>	NM.004612	1	1	1	1	7	11	5	6
<i>EDNRB</i>	NM.000115	0	1	0	1	0	1	0	2	<i>RAR-β2</i>	NM.000965	0	1	0	3	0	3	1	3
<i>GNAL</i>	NM.002071	—	—	9	19	1	4	1	4	<i>CCND2</i>	NM.001759	6	6	8	9	2	4	4	5
<i>CDKN2A</i>	NM.058195	11	3	14	15	18	9	2	3	<i>CSPG2</i>	NM.004385	4	3	3	1	0	1	1	0
<i>CDH1</i>	NM.004360	0	0	0	2	2	6	0	2	<i>GATA3</i>	NM.002051	4	4	4	4	13	10	1	0
<i>TUBA1</i>	NM.006000	4	9	9	15	8	14	6	10	<i>NME1</i>	NM.000269	1	3	2	5	2	8	1	2
<i>TIMM8A</i>	NM.004085	6	6	5	6	0	0	2	3	<i>POU3F1</i>	NM.002699	0	0	14	15	23	22	5	5
<i>CD44</i>	NM.000610	2	2	6	8	4	4	6	9	<i>RFC1</i>	NM.002913	7	3	7	3	7	3	5	3
<i>CDH13</i>	NM.001257	5	4	0	1	2	0	3	1	<i>PAX6</i>	NM.001604	7	10	—	—	9	11	1	4
<i>THBS1</i>	NM.003246	4	3	12	13	12	13	5	4	<i>NKX2-5</i>	NM.004387	4	1	4	2	3	2	4	1
<i>MUC2</i>	U67167	—	—	2	12	0	2	0	2	<i>30ST3B*</i>	NM.006041	5	3	3	1	12	11	4	3
<i>SERPINB5</i>	NM.002639	1	0	0	1	1	0	1	0	<i>CFTR</i>	NM.000492	5	6	5	4	5	6	3	3
<i>COL9A1</i>	NM.078485	0	1	0	0	1	5	1	3	<i>ABCB1</i>	NM.000927	1	0	1	0	1	0	1	0
<i>CASP8</i>	NM.033357	0	2	0	2	0	1	0	1	<i>SLC5A5</i>	NM.000453	—	—	0	1	1	2	2	3
<i>GPC3*</i>	NM.004484	12	13	13	13	13	13	10	6	<i>MAGEA1</i>	NM.004988	0	2	1	1	16	13	12	11
<i>MT1A</i>	K01383	3	7	3	7	3	7	2	3	<i>ABO</i>	NM.020469	1	2	1	1	16	13	12	11
<i>GJB2</i>	NM.004004	0	2	0	2	0	1	0	1	<i>TP53</i>	NM.000546	0	0	5	2	5	5	4	5
<i>MME</i>	NM.007288	7	8	7	7	7	7	7	7	<i>HIN-1</i>	NM.017493	0	1	1	4	1	5	5	5
<i>PLS3</i>	NM.005032	0	1	0	1	0	1	0	1	<i>KL*</i>	NM.004795	0	1	0	1	13	9	3	1
<i>SYK</i>	NM.003177	6	7	15	14	2	5	15	14	<i>ARH1</i>	NM.004675	0	1	0	1	0	2	3	0
<i>SEMA3B</i>	NM.004636	0	2	—	—	0	4	0	0	<i>TP73</i>	NM.005427	—	—	—	—	3	2	11	8

*Selected for MTA analysis.

The definition of a CpG island fragment (0.2 to 1.0-kb) is based on its GC content (>50%) and CpG score (>0.6). Frequency of methylation-sensitive sites flanked by four types of endonucleases, *MseI*, *Tsp509I*, *NlaIII*, and *Bfal*, located near or within a CpG island fragment (information derived from a BLAST search) were analyzed. "—" indicates the flanking cut sites are not present. B (*Bst*UI) and H (*Hpa*II) are internal methylation-sensitive sites.

were exposed in a PhosphorImager (Amersham Biosciences) for 1 to 7 days. Each MTA experiment was repeated 1 or 2 times. Positive hybridization signals, which are indicative of CpG island methylation in targets, were first visually scored. Because signal intensities of hybridized targets varied due to different amounts of DNA dotted on the membrane, individual spots were quantified using ImaGene 4.0 (BioDiscovery, Los Angeles, CA). The signal intensity of each spot was then normalized with that of an internal control (ie, the Cot-1 hybridized spot). A positive signal for CpG island methyl-

ation was again scored when a cutoff intensity [(signal – background) × normalized factor] was greater than 30 (an arbitrary unit) in duplicate spots of an arrayed target. Using this cutoff, the level of concordance between the two scoring methods was ≥95%.

Methylation-Specific PCR

Genomic DNA (1 to 2 μg) was treated with sodium-bisulfite according to the manufacturer's recommenda-

Figure 1. A: Methylation-sensitive restriction test. The efficiency of digestion in CpG fragments in *BRCA1* and *p16^{INK4}* was examined by subjecting duplicate tubes of genomic DNA, one doubly-digested with *Bst*UI and *Hpa*II (D) and the other mock-digested without enzymes (U). Both types of DNA were amplified under identical conditions for 17 to 35 cycles and the resultant products separated on 1.5% agarose gel. The product bands are visualized with ethidium bromide. A molecular weight ladder (M) is shown on left. A differential presence of PCR products was clearly demonstrated between the digested templates and the mock-digested templates in these two genes. **B:** Amplification of gene-specific methylation fragments in breast cancer. Pooled genomic DNA from 20 to 30 normal (N) controls or 20 to 30 breast tumors (T) was digested with a 4-base frequent cutter, ligated to end linkers, and further treated with methylation-sensitive *Bst*UI and *Hpa*II (see detailed description in the text). Subsequent PCR (20 cycles) was conducted with primers specific for promoter CpG islands (listed at top). PCR products were separated on 1% to 1.5% agarose gels. Genomic fragments containing methylated sites were protected from the digestion and could be amplified, while fragments containing unmethylated sites were restricted away and not present in the amplified samples.

tions (CpGenome; Intergen, Purchase, NY) and amplified with specific *p16^{INK4}* primers (Table 1) for methylated or unmethylated DNA. All PCR reactions were performed on PTC-100 thermocyclers (MJ Research, Watertown, MA)

Figure 2. The content of repetitive sequences in different methylation targets. **A:** Serial dilution of different cutter-generated (*Mse*I, *Tsp*509I, *Nla*III, or *Bfa*I) methylation targets (15 to 500 ng) and an identical amount of human Cot-1 DNA were dotted on the nylon filter and hybridized with radiolabeled Cot-1 probe. **B:** The hybridization signals shown in **A** were quantified by a densitometer and subsequently standardized by its respective Cot-1 spots, as discussed in the text.

Figure 3. Map locations of restriction sites in the 10 genes analyzed by MTA. The shaded horizontal bar indicates that the first exon region and its position designation is relative to the transcription start site (+1). The short vertical bars indicate the flanking 4-base endonuclease recognition sites of *Nla*III (N), *Tsp*509I (T), *Bfa*I (Bf), and *Mse*I (M), and the methylation-sensitive *Hpa*II (H) or *Bst*UI (B) sites in between. The horizontal line with flanking black circles represents the area analyzed by methylation-specific PCR. The thick horizontal line denotes the probe location for methylation target array analysis. Note that less GC-rich regions within the selected fragments were chosen as probes to prevent non-specific hybridization. The thin horizontal line under the schematics on right represents the distance of 100-bp.

and in 25- μ L volumes using the AmpliTaq system (Applied Biosystems, Foster City, CA). PCR products were separated on 1.0% agarose gels.

Demethylation Treatment and Northern and Reverse Transcription (RT)-PCR Assays

Breast cancer cell lines were cultured in the absence or presence of 5-aza-2'-deoxycytidine (DeoxyC, 750 nmol/L) for 6 days and harvested for RNA isolation using the RNeasy system (Qiagen). Ten μ g of RNA from treated and untreated samples were electrophoresed on a 1.5% agarose gel and subjected to northern analysis using a *3OST3B* cDNA probe as described.⁵ RT-PCR was conducted using primers (Table 1) located at the 3'-ends of *3OST3B*. The levels of *3OST3B* mRNA were normalized with the level of β -actin mRNA.

Statistical Analysis

A comparison of methylation frequencies across clinico-pathological parameters was performed using the χ^2 test

Figure 4. A: Methylation Target Array (MTA). The MTA chart is used to indicate the location of each arrayed target. T, tumor; N, normal control; Pos, positive control, ie, target prepared without methylation-sensitive restriction; and Neg, negative control, ie, water. T77 to T80 represent targets prepared from breast cancer cell lines ZR-75, T47D, MDA-MB-468, and Hs578t (see detailed description in the text). Shown here are the results of MTA filters hybridized with radiolabeled *p16^{INK4}* and Cot-1. Samples marked by **triangle** indicate DNA hypermethylation independently confirmed by methylation-specific PCR (MSP). Except for T23, the **asterisks** indicate no methylation detected by MSP. **B:** Methylation-specific PCR (MSP). PCR of bisulfite-treated genomic DNA was amplified with a methylated pair of primers (M) or an unmethylated pair of primers (U). Positive (+) and negative (–) controls were the Universal Methylated DNA (Intergen), respectively. PCR products were separated on 1% to 1.5% agarose gels. Breast tumors showing hypermethylation are marked in *italics*, while the remaining tumors show no methylation. **C:** Representative results of methylation target array (MTA) analysis. The restriction map of each gene is shown in Figure 3. Methylation targets were prepared as described in the text and hybridized with a ³²P-labeled probe. Positive hybridization signals, which are indicative of DNA methylation for a specific gene, are presented in each MTA panel. The grid location of each arrayed target is the same as presented in **A**.

or Fisher's exact method. The Mann-Whitney *U* nonparametric test was used to compare the coordination of DNA methylation in multiple CpG island loci. A *P* value less than 0.05 was defined as being statistically significant.

Results and Discussion

Rationale and Strategy of MTA

An initial study was conducted to determine whether the MTA strategy would prove feasible to assess CpG island hypermethylation in multiple breast tumors. To prepare targets, we restricted DNA samples with 4-base frequent cutters that cut elsewhere in the genome (<0.2-kb), but preserve as much as possible the integrity of CpG islands. Four such endonucleases, *MseI* (T↓TAA), *Tsp509I* (↓AATT), *NlaIII* (CATG↓), and *BfaI* (C↓TAG), whose recognition sequences were shown to be infrequent within GC-rich fragments,¹¹ are ideal for use in the digestion of the genome. We independently surveyed 100 genes frequently hypermethylated in various cancers and showed that DNA fragments (0.2- to 2.0-kb) restricted by these endonucleases are expected to encompass GC-rich sequences of these genes (Table 2). After

digestion, the flanking cut sites of GC-rich fragments were ligated to linkers and then sequentially restricted with 2 methylation-sensitive endonucleases, *Bst*UI and *Hpa*II, the recognition sites of which are always present in CpG islands (see examples in Table 2). The digest was used as template for PCR amplification with a linker-primer. DNA fragments containing methylated recognition sites were protected from the restriction and were amplified by PCR, while unmethylated or partially methylated fragments were cut away and could not be amplified. Therefore, the MTA technique is limited to the rapid screening of genes having completely methylated restriction sites in the region of interest. Samples with loci having partial methylation in these sites will not be detected by this technique.

Incomplete methylation-sensitive digestion might occur during sample preparation, resulting in false-positive detection of DNA methylation after PCR amplification. We therefore tested the efficiency of digestion in two CpG island fragments (*BRCA1* and *p16^{INK4}*) known to be unmethylated in normal blood DNA. Primers flanking an *NlaIII* fragment of *BRCA1*, which contains 2 internal *Hpa*II and 3 *Bst*UI sites, were used for PCR amplification (see Figure 3). Primers were also designed for amplification of

Figure 5. Profiles of Methylation Target Array in breast cancer. **A:** The methylation status of 10 promoter CpG islands in 93 breast tumors, 4 breast cancer cell lines, and 20 normal breast tissues. A **filled box** indicates the presence of methylation and an **open box** indicates the absence of methylation. CpG island loci are indicated above each column. The order of cell lines is arranged from top to bottom - T47-D, MDA-MB-231, ZR75-1, and Hs578t. **B:** Concurrent DNA hypermethylation was found in tumors hypermethylated in *GPC3* and *RASSF1A*: subgroup *a* had additional methylation in *HOXA5*, *WT1*, and *uPA* genes and subgroup *b* had additional methylation in *3OST3B*, *BRCA1*, and *DAPK1* genes.

an *Nla*III fragment in *p16^{INK4}*. We then determined at which cycles of PCR the amplification of undigested DNAs became apparent. As shown in Figure 1A, at ≥ 29 cycles of PCR the amplified *BRCA1* or *p16^{INK4}* fragment was seen in the digested samples in ethidium bromide-stained gels, suggesting that the false finding of DNA methylation could occur when high cycles of amplification are used in sample preparation. We therefore used fewer PCR cycles (20 cycles) in the MTA assay to prevent unwanted amplification of residual undigested DNA. This strategy was further tested by amplifying DNA fragments with specific primers derived from 9 CpG islands known to be hypermethylated in cancer (Figure 1B). As shown, methylated fragments were preferentially amplified in the pooled targets of 20 to 30 breast tumors, but not in those

of normal controls. No amplification was detected in a control CpG island, *KL*, in either tumor or control.

We next determined whether repetitive sequences might be overpopulated in the amplified target pools, leading to decreased sensitivity in the detection of uniquely methylated CpG islands in MTA. To determine the content of repetitive sequences after amplification, serial dilutions of 4 target pools (*Mse*I, *Tsp509*I, *Nla*III, and *Bfa*I) and the human repetitive Cot-1 DNA (used as a control) were equally dotted onto nylon membrane and hybridized with a Cot-1 probe (Figure 2A). Between 22% and 34% of repetitive sequences were calculated to be included in the methylation targets (Figure 2B). This amount is lower than that of the unprocessed genome (40% to 50%) and therefore would not be expected to interfere in the MTA hybridization with specific probes.

MTA Analysis of CpG Island Hypermethylation in Breast Cancer

MTA was initially used to survey hypermethylation of the *p16^{INK4}* CpG island in breast cancer. Methylation targets (*Nla*III fragments) prepared from 93 breast tumors, 4 breast cancer cell lines, and 20 normal breast tissues were arrayed onto nylon membrane and hybridized with a 219-bp *p16^{INK4}* probe (Figure 3 and Figure 4A). The nylon membrane was also hybridized with a Cot-1 control probe, which was used to determine the relative amounts of DNA dotted on the array. Positive hybridization signals, indicative of DNA methylation, were first visually scored on the MTA membrane and later verified with the calculated signal intensity of each spot after normalization with that of the Cot-1 control (see the scoring method described in Materials and Methods). The results showed that 22.6% (22 of 97) of breast tumors and cell lines were positive for methylation. None of the 20 normal samples, however, showed detectable methylation. To validate these methylation findings, we performed methylation-specific PCR (MSP) on 36 tumor samples (see representative examples in Figure 4B). Except for one tumor (T23), the MSP assay of the rest of the samples confirmed the MTA results. The discrepancy of T23 can be attributed to different methodologies used in the methylation analysis. Alternatively, it suggests a low level of false-positive findings by MTA, which may not interfere with methylation screening in a large number of tissue samples.

We therefore expanded the MTA analysis to an additional 9 genes, *RASSF1A*, *uPA*, *HOXA5*, *DAPK1*, *WT1*, *KL*, *BRCA1*, *GPC3*, and *3OST3B*. Their CpG islands are found to be located in the promoter and the first exon of the genes. Except for *3OST3B*, the 4-base cutter fragments to be analyzed in MTA are located near or within the promoter and first exon of these genes (Figure 3). The *Nla*III fragment of *3OST3B* is located ~ 2.5 -kb upstream of its first exon, a region previously cloned by the ICEAMP technique developed by us¹² for the identification of aberrantly-methylated DNAs in cancer. Representative results of MTA hybridization are shown in Figure 4C. CpG island hypermethylation was scored and the results were summarized in Figure 5A. The overall methylation fre-

Figure 6. Methylation and expression analysis of the *3OST3B* gene. Breast cancer cell lines were cultured in the absence or presence of 5-aza-2'-deoxycytidine (DeoxyC; 750 nmol/L) for 6 days and harvested for DNA and RNA isolation. **A:** Methylation-specific PCR. PCR of bisulfite-treated genomic DNA was amplified with a methylated primer (M) or an unmethylated primer (U); see Table 1 for primer information. Positive (+) and negative (-) controls were the Universal Methylated DNA (Intergen) and blood, respectively. PCR products were separated on 1% to 1.5% agarose gels. **B:** Reverse-transcription PCR. PCR was conducted using primers (see Table 1) derived from the 3'-end of *3OST3B*. The levels of *3OST3B* mRNA were normalized with the level of β -actin mRNA. **C:** Northern blot analysis. Ten μ g of RNA from DeoxyC-treated and untreated samples were electrophoresed on a 1.5% agarose gel and subjected to Northern analysis using a *3OST3B* cDNA probe.

Table 3. Association of CpG Island Hypermethylation with Clinicopathological Features of Patients with Breast Cancer

Gene loci	Total number of patients												
	ER/PR			Age at diagnosis			Histology			Clinical stage			
	+/+	+/-, -/+	-/-	≤50	50-70	≥70	WD	MD	PD	I	II	III	IV
GPC3													
Unmet	25	5	7	10	10	17	4	28	3	13	15	4	5
Met	24	13	19	23	21	12	5	29	22	7	32	13	4
P value	NS (0.065)			0.044			0.006			0.025			
RASSF1A													
Unmet	21	5	13	13	13	13	4	23	11	11	18	6	4
Met	28	13	13	20	18	16	5	34	14	9	29	11	5
P value	NS (0.334)			NS (0.911)			NS (0.940)			NS (0.575)			
HOXA5													
Unmet	35	9	21	27	16	22	6	40	17	16	32	10	7
Met	14	9	5	6	15	7	3	17	8	4	15	7	2
P value	NS (0.086)			0.022			NS (0.966)			NS (0.513)			
WT1													
Unmet	39	9	22	27	19	24	6	44	18	18	33	12	7
Met	10	9	4	6	12	5	3	13	7	2	14	5	2
P value	0.019			NS (0.087)			NS (0.744)			NS (0.361)			
uPA													
Unmet	37	7	23	26	19	22	6	40	19	16	30	13	8
Met	12	11	3	7	12	7	3	17	6	4	17	4	1
P value	0.001			NS (0.255)			NS (0.819)			NS (0.303)			
p16^{INK4}													
Unmet	42	14	19	28	23	24	9	44	20	19	39	10	7
Met	7	4	7	5	8	5	0	13	5	1	8	7	2
P value	NS (0.395)			NS (0.526)			NS (0.280)			0.045			
3OST3B													
Unmet	40	13	10	15	21	27	8	40	13	20	31	7	5
Met	9	5	16	18	10	2	1	17	12	0	16	10	4
P value	<0.001			<0.0005			NS (0.093)			0.001			
BRCA1													
Unmet	42	14	16	24	26	22	7	48	16	18	37	11	6
Met	7	4	10	9	5	7	2	9	9	2	10	6	3
P value	NS (0.058)			NS (0.550)			NS (0.126)			NS (0.259)			
DAPK1													
Unmet	49	17	19	29	28	28	9	56	18	19	44	14	8
Met	0	1	7	4	3	1	0	1	7	1	3	3	1
P value	<0.001			NS (0.462)			<0.001			NS (0.482)			
KL													
Unmet	49	18	26	33	31	29	9	57	25	20	47	17	9
Met	0	0	0	0	0	0	0	0	0	0	0	0	0
P value	N/A			N/A			N/A			N/A			

ER, estrogen receptor; PR, progesterone receptor; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; Unmet, unmethylated; Met, methylated.

Figure 7. Correlation of CpG island hypermethylation with clinicopathological features of breast tumors. The x axis indicates the total number of hypermethylated loci (low for 0 to 2 loci, median for 3 to 4 loci and high for 5 to 7 loci). **A:** Correlation with ER/PR status. **B** and **C:** Correlation with clinical stages and age at diagnosis, respectively. The statistical *P* values are also shown in each panel.

quencies detected in breast tumor and cell lines were 60% for *GPC3*, 58% for *RASSF1A*, 32% for *3OST3B*, 30% for *HOXA5*, 28% for *uPA*, 25% for *WT1*, 23% for *BRCA1*, and 9% for *DAPK1*. Methylation of the *KL* CpG island in these tumors was not detected by MTA. Eighty-seven percent (81 of 93) of these tumors had hypermethylation in at least one of the 10 genes studied. Furthermore, hypermethylation of multiple genes (5 to 7 loci) was observed in 20% of the tumors. The 4 breast cancer cell lines had an overall higher frequency of hypermethylation in these genes than that of primary tumors. While only one normal sample showed methylated *BRCA1* promoter, the rest of the 19 normal controls had no detectable hypermethylation by MTA.

These methylation findings are reminiscent of those previously reported in breast cancer. The high frequencies (>50%) of CpG island hypermethylation detected in *GPC3* and *RASSF1A* genes are consistent findings in breast cancer.^{7,13,14} On the other hand, hypermethylation of the *DAPK1* gene is less frequent (<20%) in breast tumors, at a rate similar to those reported.^{4,15} The frequency (23%) of *BRCA1* hypermethylation reported here is somewhat higher than that (13%) of a previous survey.⁴ The median rates of hypermethylation in *WT1*

and *p16^{INK4}* genes are also close to the reported incidence in breast cancer.^{4,16} While CpG island hypermethylation in *HOXA5* was previously reported in 80% of p53-negative breast tumors,¹⁷ the overall rate of methylation shown here is 30%. The methylation frequency of *uPA* has previously not been reported in primary tumors, but hypermethylation of this gene was shown to result in transcriptional silencing in breast cancer cell lines.¹⁸

Hypermethylation of the upstream region of *3OST3B* is a novel finding in breast cancer. This gene encodes 3-O-sulfotransferases known to modulate the sulfonation of heparan side chains needed in a wide range of biological processes.¹⁹ We further correlated this aberrant methylation with the transcriptional activity of *3OST3B* in breast cancer cell lines (Figure 6, A and B). Hypermethylation of the *3OST3B* promoter was detected in MDA-MB-231 and expression analyses revealed that this hypermethylation was associated with down-regulation of *3OST3B*, and was reactivated on the treatment of a demethylation agent, DeoxyC. The expression of *3OST3B* was also restored in breast cancer cell line T47-D (Figure 6C). No methylation of *3OST3B* was detected in ZR-75 and Hs578t (data not shown) and their expression levels of *3OST3B* were not influenced by the demethylation treatment. Our future studies will focus on methylation analysis of this gene promoter and the functional consequences of its methylation-mediated silencing in relation to breast tumorigenesis.

Association of Hypermethylation Profiles with Clinicopathological Features of Patients

The frequencies of CpG hypermethylation in the primary breast tumors analyzed were found to be associated with several clinicopathological features of the patients (Table 3). Hypermethylation of multiple loci (ie, 5 to 7 loci) was significantly correlated with ER/PR-negative (*P* = 0.002) or late-stage (*P* = 0.02) tumors (Figure 7, A and B). Hypermethylation in the 9 CpG islands assayed by MTA also occurred less frequently in older patients (≥70 years old) with breast cancer (*P* = 0.025) (Figure 7C).

This observation also supports the notion that concurrent hypermethylation of multiple CpG islands is commonly seen in breast cancer.^{7,20} Several studies have described the existence of one or more methylator phenotypes, in which hypermethylation of multiple loci simultaneously occurs in various types of cancer.^{21–23} Although the presence of a methylator phenotype could be attributed to a generalized deregulation of methylation control in the genome, our observation suggests that this event is not random in breast cancer; some promoter CpG island loci are seemingly more susceptible to this epigenetic alteration.²⁴ In this regard, we identified two loci, *GPC3* and *RASSF1A*, which are concurrently hypermethylated in 40% of the breast tumors examined. Within these tumors, one subgroup (a) had additional methylation in *HOXA5*, *WT1*, and *uPA* loci, whereas the other subgroup (b) acquired frequent hypermethylation of *3OST3B*, *BRCA1*, and *DAPK1* genes (see Figure 5B). These methylation patterns seem to imply that DNA meth-

ylation may be progressively accumulated in different CpG island loci during tumor development. Future profiling of MTA data using various clustering algorithms will be useful in identifying different epigenetic pathways for molecular classification of breast cancer subtypes.

Concluding Remarks

We have demonstrated that MTA is a candidate gene approach for potential profiling of CpG island hypermethylation in hundreds of tissue genomes. This array-based method provides a new opportunity to assess clinico-pathological significance of hypermethylated CpG islands in breast tumors and other types of cancer. Unlike other approaches, such as MSP or bisulfite sequencing, which focus on the evaluation of methylation details within individual CpG island loci, MTA is confined to uncover highly methylated CpG islands in multiple clinical samples. One advantage is that, like Southern blot, a single MTA nylon filter can be repeatedly used for probing with many CpG island loci, which allows for rapid assessment of potential methylation markers that may be clinically useful in predicting the treatment outcome of a cancer patient.

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