Differential Distribution of DNA Methylation within the RASSF1A CpG Island in Breast Cancer


ABSTRACT

Aberrant DNA methylation of promoter CpG islands is associated with transcriptionally repressive heterochromatin in neoplasia. The dynamics of this epigenetic process in mediating the transition from an active to an inactive state of transcription remains to be elucidated, however. Here, we used the methylation-specific oligonucleotide microarray to map the methylation patterns of a CpG island, located within the promoter and the first exon regions of RASSF1A, in normal breast tissue controls, primary tumors, and breast cancer cell lines. Oligonucleotide pairs, spaced along the CpG island region, were designed to discriminate between methylated and unmethylated alleles of selected sites. The methylation-specific oligonucleotide data indicate that the majority of test samples show widespread methylation in the first exon of RASSF1A. In contrast, the promoter area was usually undermethylated in normal controls and in 32% of the primary tumors tested, whereas the rest of the primary tumors and breast cancer cell lines showed various degrees of methylation in the region. Methylation profiling of individual tumors further suggest that DNA methylation progressively spreads from the first exon into the promoter area of this gene. Functional analysis indicates that increased density of RASSF1A promoter methylation is associated with altered chromatin, marked by a depletion of acetylated histones and methylated histone 3-lysine 4 and an enrichment of methylated histone 3-lysine 9 in the studied area. The combination of these epigenetic modifications may engender a stable silencing of the gene in breast cancer cells. Thus, this study underscores the importance of detailed mapping of methylation patterns within a CpG island locus that may provide insights into the progressive nature of aberrant DNA methylation and its relationship with transcriptional silencing during the neoplastic process.

INTRODUCTION

The CpG island, a stretch of GC-rich DNA sequence, is frequently located at the 5′-end regulatory regions of a gene, extending from the promoter to the first exon region (1). This area of genomic sequence is subject to epigenetic modifications, including DNA methylation and histone acetylation and methylation, which are known to play an important role in regulating gene expression (2). In normal cells, the majority of promoter CpG islands are protected from this epigenetic event and are unmethylated (2, 3). In cancer cells, it has been shown that widespread DNA methylation occurs in many promoter CpG islands, resulting in a closed, repressed chromatin configuration that disables transcription initiation of the associated genes (3).

Although CpG island hypermethylation has been observed in many types of solid tumors and leukemias (4, 5), the dynamics of this epigenetic process in mediating the transition from an unmethylated, active state to a densely methylated, inactive state of a gene promoter remains largely unknown. Using cultured cells overexpressing DNA methyltransferase DNMT1, Graff et al. (6) observed that de novo methylation is a gradual process and progressively accumulated within a susceptible CpG island. The in vivo observation of this so-called methylation spread phenomenon still awaits proof in vivo. To conduct such a study in primary tumors, detailed mapping of methylated sites across a CpG island of interest is needed. The frequently used assays, methylation-specific PCR (7), or COBRA (8), which detect point methylation on only a few sites within a CpG island, may not be suitable for such a task. The bisulfite sequencing method (9) is the choice for comprehensive methylation mapping but is more labor intensive for analyzing a large panel of primary tumors representing different stages of methylation progression.

Toward this end, we recently developed an oligonucleotide-based microarray technique, MSO microarray (10), which can improve the resolution of methylation mapping in a defined CpG island region. In the MSO assay, DNA targets are hybridized to a series of arrayed oligonucleotide probes, designed to be closely spaced along an interrogating sequence. Each pair of oligonucleotides is capable of discriminating between methylated and unmethylated alleles at specific sites. Herein we used the MSO microarray to assess methylation profiles of the RASSF1A CpG island region in a panel of breast normal tissue controls, primary tumors, and cancer cell lines. This CpG island, spanning the promoter and the first exon regions of the gene, is hypermethylated in 42–65% of primary breast tumors (11–14) and in many other cancer types (15–17). Recent evidence suggests that the methylation-associated silencing of RASSF1A transcript can disrupt RAS and its related signaling pathways, leading to uncontrolled tumor growth.

The frequent observation of RASSF1A hypermethylation in breast cancer therefore provides an ideal model for examining the methylation spread phenomenon in vivo. Our MSO results demonstrate that DNA methylation is differentially distributed within the RASSF1A CpG island in breast cancer. This disparity observed in breast tumors may reflect different stages of methylation progressively spreading from the nearby first exon into the promoter of the RASSF1A gene. Additional analysis suggests that RASSF1A promoter methylation is associated with altered chromatin configuration, marked by specific patterns of histone acetylation and methylation.

MATERIALS AND METHODS

Sample Preparation, Sxl Treatment, and Bisulfite Modification. Thirty-seven breast tumors were obtained from patients undergoing mastectomy before chemotherapy at the Ellis Fischel Cancer Center (Columbia, MO), in compliance with the Institutional Review Board. Except for two invasive lobular carcinomas (T301 and T313), the rest of the tumors analyzed were classified as infiltrating ductal carcinomas. Ten tumor-free breast parenchymas were obtained to serve as controls. Breast cancer cell lines MDA-MB-435s,
DNA METHYLATION OF THE RASSF1A CpG ISLAND

Fig. 1. Genomic map of the RASSF1A CpG island. The positions of CpG sites in the genomic sequence are indicated by thin vertical lines. Bent arrow, the position of the transcription start site. Potential transcription factor binding sites (MZF1, GATA-2, AML-1a, and Sp1) resulting from the TFSEARCH query are marked in the promoter region. The names and locations of MSO pairs capable of distinguishing methylated and unmethylated alleles are presented below the RASSF1A map. The regions amplified for the COBRA are shown in thick horizontal lines with vertical arrows indicating the interrogating BstUI restriction sites (CCGG, see Fig. 3). Bisulfite-treated genomic DNA was amplified in three regions (P1, P2, and E1) and used as target for MSO hybridization. The bottom row of three horizontal lines indicates the locations of RASSF1A promoter fragments used for the ChIP assay (see Fig. 6).

ZR-75-1, HeLa, MDA-MB-453, MCF7, MDA-MB-231, and T47D were routinely maintained in our laboratory as described (18). Genomic DNA was isolated using the QIAamp Tissue Kit (Qiagen). For the preparation of 100% methylated DNA, a blood DNA sample was treated with M. SssI methyltransferase that methylates all cytosine residues of CpG dinucleotides in the genome. Sodium bisulfite modification of the test samples and M. SssI-treated DNA samples was then performed using the CpGenome DNA Modification Kit (Intergen). This treatment converts unmethylated, but not methylated, cytosine to uracil in the genome.

MSO Microarray. Preparation of MSOs was carried out essentially as described (10). These oligonucleotides were used to interrogate bisulfite-treated sense strand DNA in regions covering the promoter and first exon of RASSF1A. Each oligonucleotide (final concentration: 50 pmol/ml) was suspended in microspoting solution (Telechem) and printed in triplicate as microdots (~1 nl; 100 μm diameter) on the superaldehyde-coated glass slides (Telechem) using a GMS 417 microarrayer. Slide processing was performed following the manufacturer’s protocol (Telechem). For DNA target preparation, bisulfite-treated DNA was amplified by PCR from three regions (P1, P2, and E1) located in the RASSF1A CpG island (Fig. 1). PCR conditions were the same as those described previously (10). Primers used for bisulfite DNA amplification were: P1, sense strand, 5′-GTA GTA TAA GTG TGT TTT AGT and antisense strand, 5′-TAT CCC TTC CTT CCC TCC TT; P2, sense strand, 5′-AGG AGG GAA GGA AGG GTA AG and antisense strand, 5′-TAA CTT ACA AAC-3′; first exon sense strand, 5′-AGG TGY GGG TTY GTT TGT TGT TTT and antisense strand, 5′-GCG ACT TCA ACA AAT TA ACG TCA CAA AAA A A T. After amplification, 32P-incorporated PCR products were digested with the restriction enzyme BstUI (New England Biolabs), which recognizes sequences unique to the methylated and bisulfite-unconverted alleles. The digested and undigested control DNA samples were separated in parallel on 8% polyacrylamide gels and subject to autoradiography using a PhosphorImager (Amersham-Pharmacia).

Bisulfite Sequencing. The P2 and E1 regions (see Fig. 1) within the RASSF1A CpG were amplified from bisulfite-treated genomic DNA by PCR using the same primer pairs described earlier. Amplified products were subcloned into the TOPO-TOA Cloning System (Invitrogen). Plasmid DNA of 10–15 insert-positive clones were isolated by the Qiagenprep Spin Miniprep kit (Qiagen) and sequenced using the ABI sequencing system (Applied Biosystems).

Semiquantitative RT-PCR. Total RNA (1 μg) was extracted, reverse transcribed, and used to amplify cDNA from a 5′-end region of RASSF1A or from β-actin (control) with AmpliTaq Gold polymerase (Applied Biosystems). The primers used for PCR were: RASSF1A, sense strand, 5′-ACC TCT GTG GGG ACT TCA CCT CCT CCC TCC TT; control) in each sample.

ChIP-PCR. The assay was conducted using a kit from Upstate Biotechnology and followed the manufacturer’s protocols with some modifications. Breast cancer cells (2 × 10^5/assay) were cross-linked using 1% formaldehyde. Cell nuclei were isolated and lysed in an SDS buffer containing protease inhibitors. The samples were sonicated and preclared with salmon sperm DNA/Protein A agarose beads (Upstate Biotechnology). The soluble chromatin fraction was collected, and 8 μl of antibodies for acetyl-H3, acetyl-H4, methyl-H3-K4, or 12 μl of methyl-H3-K9, or no antibody, was added and incubated overnight with rotation. After elution from the beads, the immunocomplex was further digested with proteinase K to release bound chromatin DNA. DNA was then recovered by phenol extraction, ethanol precipitated, and resuspended in water. Primers were designed to separately amplify three regions in the RASSF1A promoter area (Fig. 1). One additional primer set was designed to amplify a 300-bp fragment of the γ-actin gene as an internal control (19). Primer sequences used for ChIP-PCR were: RASSF1A region 1, sense strand, 5′-GCT TCA GCA AAC CGG ACC AGG and antisense strand,
RESULTS

Standardization of MSO Microarray for Quantitative Methylation Analysis. A panel of 56 oligonucleotide probes were initially designed to map the methylation status of a 650-bp CpG island region, spanning the promoter and the first exon of RASSF1A. Sequences of these oligonucleotide probes were derived from the bisulfite-treated sense strand DNAs in the region. Diplex PCR for simultaneously amplifying both test and control fragments was performed using the AmpliTaq Gold polymerase (Perkin-Elmer). After 25–30 cycles of amplification, 32P-incorporated PCR products were run on 8% polyacrylamide gels and subjected to autoradiography. Densitometric analysis of the observed bands was performed using ImageQuant (Molecular Dynamics). Fold-enrichment was derived from the ratio between the intensities of test and control fragments from the bound sample to that of the total input sample.
described in our previous study (10), cross-hybridization likely occurs between imperfect match probes and targets, especially in a stretch of DNA containing too many consecutive Ts, Gs, As, or Cs or CGs. In addition, bias of PCR amplification on either the methylated or the unmethylated DNA molecules may occur in the bisulfite-treated samples, leading to an inaccurate estimate of methylation in target DNAs (21). Therefore, to test the accuracy and reproducibility of the MSO probes, targets were first prepared from a series of mixed samples containing 100, 66, 33, and 0% SssI-methylated DNAs and subjected to MSO hybridization. For an optimal probe set, levels of DNA

![Image](image_url)
methylation are expected to be proportional to the calculated intensity ratios of M/(M+H). Of the 56 (or 28 paired) oligonucleotide probes initially tested, 68% (19 paired) of the probes were capable of distinguishing methylated versus unmethylated alleles from these mixed samples. The locations of these optimal probes in the RASSF1A CpG island region are shown in Fig. 1. Standardization curves generated for these optimal probe sets were subsequently used to derive levels of RASSF1A methylation in the test DNA samples (10).

**Verification of MSO Findings by COBRA and Bisulfite Sequencing.** Next, we verified the MSO findings by COBRA, which was designed to detect DNA methylation in three BstUI sites located in the promoter region and six BstUI sites in the first exon of RASSF1A (see Fig. 1). In COBRA, bisulfite-treated DNAs were individually amplified by PCR and then treated with BstUI, which restricts CGCG recognition sites in the samples. Unmethylated sites were converted to TGTG after PCR and resisted BstUI digestion, whereas methylated sites remained unconverted and thus restricted by this endonuclease. The digested fragments displayed in gel electrophoresis are indicative of methylated BstUI sites in the test region. Fig. 3 shows representative examples of COBRA, and the results of all samples tested are summarized in parallel with the MSO findings in Fig. 2. The methylation findings by COBRA significantly corroborate those by MSO (χ² test, P < 0.001). In all but two normal controls and 32% (12 of 37) of the primary tumors analyzed, BstUI methylation was not detected in the promoter but was detected in the first exon of RASSF1A. In the rest of 21 primary tumors and all cancer cell lines, BstUI methylation was present in both regions. Four of the
primary tumors, T321, T237, T191, and T217, that fell between these two categories showed discrepancies in their promoter methylation levels as determined by MSO versus COBRA. These samples, having light methylation in the promoter region, fell into a region whereby the sharp methylation demarcation observed in the preceding samples was less distinct (see Fig. 2). These discrepancies could be attributed to the differential sensitivity of methodologies used for the detection of subtle methylation changes. In this regard, COBRA is restricted to detecting complete or partial methylation on a few BstUI sites and could not accurately reflect a quantitative measurement of DNA methylation within the survey region by MSO. Alternatively, it suggests a potential false result determined by MSO in these samples, which may have little impact on the observation of differential methylation profiles uncovered in this study.

The intent of the MSO analysis is to provide an overall assessment of DNA methylation in one to three CpG sites represented by individual oligonucleotide sets. Thus far, only the bisulfite sequencing approach is capable of determining the methylation status of every CpG site in an interrogating region. We further compared the levels of RASSF1A methylation determined by bisulfite sequencing with those obtained by MSO in five DNA samples (N212, T177, T103, T47D, and MDA-MB-435s). As shown in Fig. 5, methylation of cloned DNA strands in each sample analyzed cumulatively reflects the overall methylation profiles determined by MSO (see Fig. 2). Additionally, bisulfite sequencing data indicate that the majority of DNA strands affected by aberrant methylation were presented in an all-or-none manner. However, mosaic patterns of DNA methylation were seen in some promoter strands in T103, T47D, and MDA-MB-435s.

**Functional Analysis of the RASSF1A CpG Island in Breast Cancer Cells.** To establish a functional relationship between DNA hypermethylation and the silencing of RASSF1A transcript, semiquantitative RT-PCR was conducted in 12 primary breast tumors and 7 breast cancer cell lines. A normal tissue panel and 7 normal breast samples were used as controls for determining the basal levels of RASSF1A mRNA. As shown in Fig. 5, the expression levels of RASSF1A are significantly higher in normal breast tissue controls than those of primary tumors (P = 0.003, Mann-Whitney rank-sum test). Within the tumor group tested, although not statistically significant, we found a trend that tumors showing higher levels of RASSF1A expression usually had lower overall promoter methylation. Three tumors (T187, T185, and T293) that had high levels of promoter methylation (30–40%) exhibited a significant amount of RASSF1A transcript, which might have been attributed to the presence of residual normal cells in these samples. Except for MDA-MB-435s, this transcript was not detected in breast cancer cell lines ZR-75-1, Hs578t, MDA-MB-453, MCF7, or MDA-MB-231, which showed relatively high levels (>60%) of promoter methylation in RASSF1A by MSO and bisulfite sequencing. MDA-MB-435s differs from the remaining cell lines in that a low level (~20%) of promoter methylation was observed in this cell line. Nonetheless, high levels (60–90%) of first exon methylation were seen in all of the cell lines, including MDA-MB-435s. The detection of RASSF1A transcript by RT-PCR therefore suggests that promoter methylation, rather than first exon methylation, in this CpG island region is responsible for the silencing of this gene.

The association of promoter methylation and gene silencing in relation to chromatin remodeling has not been reported previously in RASSF1A. To establish this functional link, we focused the analysis on two breast cancer cell lines, MDA-MB-435s and T47D. As indicated earlier, the former had an overall low level of promoter methylation in RASSF1A, whereas the latter showed extensive methylation in the CpG island. We performed ChIP assays on MDA-MB-435s and...
T47D cells. The histone-associated DNAs, immunoprecipitated with antibodies against acetyl-H3, acetyl-H4, dimethyl-H3-K4 (methyl-H3-K4), or dimethyl-H3-K9 (methyl-H3-K9), respectively, DNA fragments corresponding to RASSF1A promoter regions 1, 2, and 3 (see Fig. 1) were amplified by PCR. Images on left are PCR analyses of ChIP on MDA-MB-435s and T47D in the three regions. Diplex PCR was performed on immunoprecipitated DNA and total input DNA. The corresponding signal intensity enrichment of each ChIP-PCR analysis for the three regions is plotted on the right. Enrichment levels are derived from fold-changes between the normalized RASSF1A intensity in each immunoprecipitated DNA and the normalized RASSF1A intensity in the input DNA. Note that a minor band occasionally occurred in Lane 2 directly below the amplified γ-actin fragment because of nonspecific PCR amplification.

Fig. 6. ChIP assay on the RASSF1A CpG island. Chromatin DNA was immunoprecipitated with antibodies specific for acetyl-H3, acetyl-H4, dimethyl-H3-K4, or dimethyl-H3-K9 (methyl-H3-K9), respectively. DNA fragments corresponding to RASSF1A promoter regions 1, 2, and 3 (see Fig. 1) were amplified by PCR. Images on left are PCR analyses of ChIP on MDA-MB-435s and T47D in the three regions. Diplex PCR was performed on immunoprecipitated DNA and total input DNA. The corresponding signal intensity enrichment of each ChIP-PCR analysis for the three regions is plotted on the right. Enrichment levels are derived from fold-changes between the normalized RASSF1A intensity in each immunoprecipitated DNA and the normalized RASSF1A intensity in the input DNA. Note that a minor band occasionally occurred in Lane 2 directly below the amplified γ-actin fragment because of nonspecific PCR amplification.

DISCUSSION

Aberrant DNA methylation of the RASSF1A CpG island and its associated gene silencing have been reported in many tumor types, including breast cancer (15–17). However, the dynamics of this epigenetic event in relation to breast tumorigenesis remain to be elucidated. Fine mapping of methylation patterns in the Rb (23), p15INK4B (24), GSTP-1 (25), CDKN2A (26), and TMS1 (27) CpG islands has provided useful insights into the mechanisms of aberrant DNA methylation in cancer. In this study, we used MSO to generate methylation profiles of the RASSF1A CpG island in breast normal controls, primary tumors, and breast cancer cell lines. The island includes a 650-bp region, encompassing the entire promoter and almost all of the first exon of this gene. The MSO data, which were independently confirmed by COBRA, showed that the distribution of methylated sites was not equally represented within this CpG island. The majority of the test samples (≥80%) showed widespread methylation in the first exon region. In contrast, the promoter area was usually undermethylated in normal controls and some primary tumors (32%), whereas the rest of the primary tumors showed various degrees of methylation in the region. The extensive methylation of both the promoter and the first exon observed in almost all of the breast cancer cell lines analyzed could be attributed to the long-term culture effect on these cells (28).

Our methylation profiling implicates the progressive nature of RASSF1A methylation in breast cancer. Such a view is further supported by a recent observation that RASSF1A methylation was progressively accumulated in sequential passages of human mesothelial cells transfected with SV40 (29). In addition, an earlier study reported the methylation spreading of the E-cad and VHL CpG islands in cultured fibroblasts overexpressing DNA methyltransferase DNMT1 (6). This in vitro methylation spread occurred in a time-dependent manner, likely starting from the outer flanks of the CpG islands and gradually moving toward the transcription start sites of these genes (6). The in vivo proof of this progressive model may have to come from a longitudinal study, which requires methylation analysis through a series of tumor samples obtained from the same patient.
In neoplastic cells, the border that separates the methylated first exon with the unmethylated first exon within a susceptible CpG island is associated with more advanced stages of tumors (24, 26). In this study, there was no apparent concurrent methylation of multiple CpG islands linked to more advanced cancer patients, likely attributable to the smaller sample size used in the study.

One possibility is that a decrease in the occupancy of transcription factors on one of the putative Sp1 [(T/G)(A)/GGC/(T/G)(G/A)/(G/A) (C/T)] sites, located within ~10-bp upstream of the transcription start site, renders an easy access of DNA methyltransferase to CpG sites located in the promoter. Disruption of the Sp1 elements has been implicated previously as a factor in de novo methylation in gene promoters (6, 31, 32). The process may be slow, resulting in a subtle decrease in the level of RASSF1A transcription. This decrease in transcription might then foster an additional decrease in the protection of this CpG island, allowing for additional methylation to accumulate in the promoter area. Increased density of DNA methylation in the promoter may attract the binding of methyl-CpG-binding proteins, such as MeCP2 and other repressors, which may directly interact with histone methyltransferases or deacetylases, resulting in an alteration of the histone code (3). As evidenced by our functional analysis, densely methylated promoter in T47D cells is tightly linked to a depletion of acetylated histones and methyl-H3-K4 and an enhancement of methyl-H3-K9, in the studied area. In contrast, the active promoter in MDA-MB-435s cells reveals an inverse pattern of these chromatin modifications. Our data therefore indicate that aberrant DNA methylation, in partnership with a combination of histone modifications, sets up a repressive heterochromatin to establish stable silencing of the RASSF1A gene in breast cancer cells.

Recent evidence has indicated that increased density of methylation within a susceptible CpG island is associated with more advanced stages of tumors (24, 26). In this study, there was no apparent statistical association of methylation progression in RASSF1A with clinicopathological parameters (see Fig. 3, right panel) of breast cancer patients, likely attributable to the smaller sample size used in this study. Alternatively, because there is also an indication that concurrent methylation of multiple CpG islands is linked to more malignant features of tumors (33, 34), the clinicopathological association may become more obvious when the progression profiles of DNA methylation are simultaneously analyzed in multiple loci by MSO in future studies.

In conclusion, detailed mapping of methylation patterns of susceptible CpG islands in cancer cells is increasingly important for epigenetic analysis. Ideally, the bisulfite sequencing approach should be used, yielding methylation levels of every CpG site within a given locus. Here, we have shown that MSO microarray, which can survey DNA methylation in the genomic sequence of interest at a high-throughput manner, is an effective alternative to bisulfite sequencing. Such a thorough analysis is expected to provide insights into the progressive nature of aberrant DNA methylation and its relationship to transcriptional silencing in the neoplastic process.

ACKNOWLEDGMENTS

We thank Diane Peckham and Deiter J. Duff for their technical support and Dr. Christoph Plass for critical reading of this manuscript.

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