Identification and Characterization of Differentially Methylated Regions of Genomic DNA by Methylation-sensitive Arbitrarily Primed PCR

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Abstract

We have developed a simple and reproducible fingerprinting method for screening the genome for regions of DNA that have altered patterns of DNA methylation associated with oncogenic transformation. Restriction enzymes with different sensitivities to cytosine methylation in their recognition sites were used to digest genomic DNAs from primary tumors, cell lines, and normal tissues prior to arbitrarily primed PCR amplification. Fragments that showed differential methylation were cloned and sequenced after resolving the PCR products on high-resolution polyacrylamide gels. The cloned fragments were then used as probes for Southern analysis to confirm differential methylation of these regions in colon tissues and cell lines. Forty-four DNA fragments associated with a total of five different regions of genomic DNA containing methylation sites were detected in 10 matched sets of normal and tumor colon DNAs and 7 colon cancer cell lines. A novel CpG island was also isolated that was found to be frequently hypermethylated in bladder and colon tumors. We have demonstrated that this technique is a rapid and efficient method that can be used to screen for altered methylation patterns in genomic DNA and to isolate specific sequences associated with these changes.

Introduction

Approximately 1% of cytosines in vertebrate DNA are methylated at CpG dinucleotides (1). The presence of 5-methylcytosine at CpG dinucleotides may contribute to tumorigenesis either by generating point mutations or by altering gene expression (2, 3). DNA methylation has been shown to be essential for normal embryonic development (4), and alterations in both the levels and patterns of methylation during transformation may affect the normal regulation of gene expression (3, 5). The number of CpG dinucleotides in the human genome is underrepresented by a factor of 5 (6), presumably due to the conversion of methylated cytosine to thymine via deamination (7); however, certain areas of the genome do not show such suppression, and these areas are known as CpG islands (1, 8, 9). CpG islands are commonly associated with housekeeping genes and may regulate their transcriptional activities. Hypomethylation of these regions is usually associated with gene activity, whereas methylation of CpG islands such as those on the inactive X chromosome (10) may suppress transcription. Methylation of CpG islands typically occurs only in parentally imprinted genes (11, 12) and in genes that have been silenced on the inactive X chromosome. Autosomal CpG islands in nonimprinted genes remain unmethylated in the normal state but may become methylated upon oncogenic transformation (13, 14). It has been demonstrated previously that alterations in the methylation status of CpG islands during transformation are correlated with transcriptional changes in a number of genes associated with growth regulation (15–17), and it is possible that other genes involved in cell cycle control may also be influenced by these epigenetic mechanisms.

The ability to detect methylation changes associated with oncogenic transformation is of critical importance in understanding how DNA methylation may contribute to tumorigenesis. A variety of techniques have been previously developed that can be used to investigate patterns of DNA methylation, such as genomic sequencing (18, 19), PCR-based methylation analysis (20), and Southern blotting (21). However, a fundamental limitation of both genomic sequencing and PCR-based methylation analysis is that these methods require some knowledge of the DNA sequence being studied. Techniques including Southern blotting, high-performance liquid chromatography analysis (22), and the methyl-acceptance assay (23) have been used to study global levels of DNA methylation. None of these methods, however, can be used to isolate specific and unknown DNA sequences from genomic DNAs that are differentially methylated between normal and tumor tissues. RLGS (24) can be used to identify specific methylation differences between genomes; however, this technique is rather labor-intensive, and deletions, amplifications, and rearrangements may also be resolved. AP-PCR has been used previously to identify genetic changes including loss of heterozygosity and chromosomal gains in colorectal tumors (25, 26). The application of this method for identifying and characterizing methylation differences between genomes, however, has not been demonstrated. We describe a methylation-sensitive AP-PCR technique that uses methylation-sensitive restriction digestion coupled with AP-PCR to identify random but specific methylation changes at multiple sites in genomic DNA in a rapid and efficient manner.

Materials and Methods

DNA Isolation from Tissues and Cell Lines. Matched pairs of normal and tumor colon and bladder specimens were obtained from patients treated at the Los Angeles County-University of Southern California Medical Center and the University of Southern California/Norris Comprehensive Cancer Center (Los Angeles, CA). Mucosal tissue was first removed from surrounding muscle and fat, and DNA was then isolated using standard procedures by treatment with proteinase K and phenol extraction (27). Seven colon cancer cell lines (SW837, SW480, HT-29, LoVo, SW48, HCT 116, and HCT-15) were obtained from the American Type Culture Collection and grown in the appropriate medium as suggested by the American Type Culture Collection. DNA was isolated from these cell lines in the same manner as described previously (27).

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6 The abbreviations used are: RLGS, restriction landmark genomic scanning; AP-PCR, arbitrarily primed PCR.
Restriction Enzyme Digestion of Genomic DNA. Two µg each of normal
and tumor DNA from tissues or DNA from colon cancer cell lines were
separately digested with either 20 units of Rsal, 20 units each of Rsal and
the methylation-sensitive restriction enzyme HpaII, or 20 units each of Rsal and
MspI (Boehringer Mannheim, Indianapolis, IN) at 37°C for 16 h. Double
restriction enzyme digestions were used to reduce the number of PCR frag-
ments and potential artifacts that might be amplified in the AP-PCR. HpaII
does not cut DNA if the internal cytosine of its restriction site (CCGG) is
methylated, whereas MspI is insensitive to the methylation status of the
internal cytosine. Restriction enzymes were heat inactivated by incubating
the reactions at 65°C for 20 min and then stored at −20°C.

Methylation-sensitive AP-PCR. Restriction-digested DNA (200 ng) was
amplified using AP-PCR (25, 26) with a single primer (MLG2: AAC CTC
CAC CTC AAC CGG G)(or a combination of 2 primers (MGC0: AAC CTC
CAC CTC AAC CGG G) + MGF2: AAC CTC AAC CTC AAC CCG CG).
PCR reactions were performed in a total volume of 25 µl under the following
conditions: 10 mm Tris-HCl (pH 8.3), 1.5 mm MgCl2, 50 mm KCl, 0.1% gelatin
per ml, 200 µm each of the four deoxyribonucleotide triphosphates, 2 µCi
of 32P-labeled dATP, 25 pmol of primer, and 0.8 units of Taq polymerase
(Boehringer Mannheim). Five cycles at low-stringency conditions of 94°C for
30 s, 40°C for 60 s and 72°C for 30 s were done before increasing the stringency
of the PCR to 94°C for 15 s, 55°C for 15 s, 72°C for 60 s for another 30 cycles.
PCR products were resolved on high-resolution 5% polyacrylamide gels
derenaturing conditions (7 M urea), dried, and exposed to autoradiographic film
(Amersham Corp., Cleveland, OH) overnight. Sequence data for all DNA
fragments described in this paper have been submitted to GenBank (accession
numbers: Y10088—Y10093).

Isolation and Sequencing of DNA Fragments. Candidate bands that
appeared to be differentially methylated by AP-PCR analysis were excised
from dried polyacrylamide gels and placed in a microcentrifuge tube contain-
ing 50 µl of sterile H20. The microcentrifuge tube was heated to 80°C for 10
min and vortexed to facilitate dissolution of DNA. The eluate (0.5 µl) was
then used in a PCR reaction with the same primer(s) used in the original AP-PCR
to generate sufficient amounts of template for plasmid cloning and sequencing.
PCR ingredients were the same as previously described with the following
amplification parameters for a total of 30 cycles: 94°C for 60 s, 55°C for 30 s,
72°C for 90 s were done before increasing the stringency of the PCR to 94°C for
15 s, 55°C for 15 s, and 72°C for 60 s for another 30 cycles. AP-PCR products
were considered to contain one or more CCGG sequences that were differentially
methylated between normal and tumor colon DNAs.

Results

Methylation-sensitive AP-PCR Identifies Differences between
Normal and Tumor Colon Samples and Cell Lines. Restriction
enzyme digestions with Rsal, Rsal + HpaII, and Rsal + MspI were
used as a rapid and efficient means of preparing DNA for methylation-
sensitive AP-PCR analysis. The Rsal restriction enzyme was used in
addition to HpaII and MspI to generate smaller fragments of DNA
prior to AP-PCR and to reduce the number of potential artifacts that
might be generated. AP-PCR was performed on digested DNA using
a single 20-mer primer (MLG2) under low-stringency conditions. The
MLG2 primer was designed with a 3’ end complementary to the
recognition sequence of HpaII and MspI. The possible formation of
primer dimers produced as a result of the interaction between the
palindromic 3’ ends of the primers (-CCGG) did not inhibit the
amplification of a large number of DNA fragments (Fig. 1). No
PCR products would be produced if the region of DNA between two
primer annealing sites contained at least one unmethylated HpaII site
or if at least one of the primer annealing sites contained an
unmethylated CCGG sequence. DNA digested with Rsal only and DNA
digested with Rsal + MspI served as controls for determining whether
bands observed in the AP-PCR of Rsal + HpaII digested DNA were
due to differential methylation of CCGG sequences within the region
of amplification. AP-PCR products were considered to contain one or
more CCGG sites if DNA digested with only Rsal produced a PCR
product and if Rsal + MspI digestion yielded no PCR product
because MspI should cut its recognition site regardless of the meth-
ylation status of the internal cytosine and prevent DNA amplification.

Fig. 1A shows DNA bands generated by methylation-sensitive
AP-PCR on matched pairs of normal and tumor colon DNAs. At least
five fragments (A–E) of different sizes were present in Rsal digests
of normal and tumor DNAs, absent in Rsal + MspI digestions of both
normal and tumor DNAs, present in most of the Rsal + HpaII
digestions of normal DNAs, and absent in Rsal + HpaII digestions of
malignant tumor samples. These fragments suggested that these bands were
hemimethylated in colon tumor DNA compared to normal colonic
epithelium. Fragments were excised from dried polyacrylamide gels,
reamplified, cloned, and sequenced. Interestingly, none of these frag-
ments contained HpaII sites located between the primer annealing
sites. The absence of internal HpaII sites in fragments A–E suggested
that one or both of the primer annealing sites contained CCGG
sequences that were differentially methylated between normal and
tumor DNA.

Methylation-sensitive fingerprints for seven colon cancer cell lines
are shown in Fig. 1B. All of the major PCR products previously
identified in the AP-PCR analysis of normal and tumor colon DNAs
were also identified in this separate experiment. All five AP-PCR
fragments that were previously identified as being hemimethylated
in colon tumor DNA compared to normal colon tissue were also
found to be unmethylated in some of the colon cancer cell lines examined.
Each of these AP-PCR bands was isolated, and all fragments were
confirmed to be the same PCR products that were previously identi-
fied in the AP-PCR gel of normal and tumor colon DNAs by sequence
analysis. A total of 44 DNA fragments associated with approximately
five different regions of genomic DNA containing methylation sites
were identified by methylation-sensitive AP-PCR in 10 matched sets
of normal and tumor colon tissues and 7 colon cancer cell lines.
Of these fragments, 27 (61%) were differentially methylated in
normal and tumor DNAs and 17 (39%) were found to be unmethylated
in different colon cancer cell lines.

Identification of Novel CpG Island Hypermethylation in Tu-
mors by Methylation-sensitive AP-PCR. We also used a combina-
tion of 2 primers (MGC0 + MGF2) in the methylation-sensitive
AP-PCR technique to screen for methylation differences in matched
pairs of normal and tumor colon and bladder samples. Fig. 2 shows
bands generated by AP-PCR, which amplified a region of DNA that
was hypermethylated in tumors compared to normal tissues. Banding
patterns suggested that this region of DNA, which was approximately
580 bp in length, was hypermethylated in three of six (50%) colon
tumors and three of seven (43%) bladder tumors. Further analysis of
this band revealed the presence of two internal HpaII sites, a G+C
content of 57%, and an observed/expected CpG ratio of 0.62, which
fulfills the sequence criteria for being a CpG island (9). GenBank data
base searches produced no significant matches with any known se-
Fig. 1. A, methylation-sensitive fingerprints of matched pairs of normal and tumor colon samples using primer MLG2 in the AP-PCR reactions after a 16-h restriction enzyme digestion with 20 units each of RsaI, RsaI + HpaII, or RsaI + MspI. Bands that appeared to be differentially methylated between matched normal and tumor samples are indicated by arrows. N, normal DNA; T, tumor DNA. B, methylation-sensitive fingerprints of colon cancer cell line DNAs using primer MLG2 in the AP-PCR reactions after a 16-h restriction enzyme digestion with 20 units each of RsaI, RsaI + HpaII, or RsaI + MspI. Bands that appeared to be unmethylated in cell lines are indicated by arrows. Major band groups that were isolated and sequenced are indicated (A–E). R, RsaI digestion; H, RsaI + HpaII digestion; M, RsaI + MspI digestion.

quences. Our findings demonstrate that methylation-sensitive AP-PCR can therefore be used for the identification of novel CpG islands that may become differentially methylated during tumorigenesis.

Purity of Fragments Isolated from AP-PCR Gels. Fragments that were of potential interest were excised from the AP-PCR polyacrylamide gels and reamplified before cloning for DNA sequence analysis to assess their purity. The five different PCR fragments previously identified as being differentially methylated in both primary tissues and colon cancer cell lines were excised and reamplified with the same primer used in the original AP-PCR reactions. Multiple plasmid clones were sequenced from each of the bands isolated from the AP-PCR gels of colon cell line DNAs. These results indicated the presence of only one species of PCR product for each of the isolated fragments based on sequence analysis of multiple plasmid clones.

Southern Analysis Confirms Differential Methylation. Confirmation of the potential methylation differences identified by methylation-sensitive AP-PCR by another technique was necessary to support the validity of the assay. Southern analysis was performed to determine whether PCR fragments representing putative hypomethylated regions of DNA in colon tumors and cell lines resulted from
Fig. 2. Methylation-sensitive fingerprints of matched pairs of normal and tumor colon and bladder samples using primers MGCO + MGF2 in the AP-PCR reactions after a 16-h restriction enzyme digestion with 20 units each of Rsal, Rsal + HpaII, or Rsal + MspI. Bands that appeared to be differentially methylated between matched normal and tumor samples are indicated by arrows. N, normal DNA; T, tumor DNA; R, Rsal digestion; H, Rsal + HpaII digestion; M, Rsal + MspI digestion.

authentic methylation changes. Several of the AP-PCR fragments identified by this technique did not contain internal HpaII sites as determined by sequence analysis, and it was believed that the methylation changes observed on the AP-PCR gels were due to changes in methylation of putative CCGG site(s) to which the primers may have annealed. Fig. 3A shows the results obtained from Southern analysis of three matched sets of normal and tumor colon DNAs previously analyzed by AP-PCR (Fig. 1). These samples were digested with either Rsal, Rsal + HpaII, or Rsal + MspI and plasmid cloned fragment A was used as a probe for hybridization to these DNAs. The Southern blot clearly showed bands of strong intensity at approximately 1500 bp in normal and tumor DNA digested with Rsal only, absence of a strong hybridization signal at 1500 bp in tumor samples digested with Rsal + HpaII (patients 1 and 5), and presence of a band at 1500 bp in all of the matched normal colon DNAs. A 1500-bp band was present in the Rsal + HpaII-digested lane of colon tumor DNA for patient 4. All normal and tumor DNAs digested with Rsal + MspI produced hybridization signals at approximately 900 bp. Banding patterns were consistent with the hypomethylation of the genomic DNA region associated with fragment A in at least 66% (two of three) of these colon tumors compared to normal tissues.

Southern analysis also showed similar hybridization patterns for colon cancer cell lines probed with band A (Fig. 3B). Absence of a 1500-bp band and presence of a 900-bp band in the Rsal + HpaII-digested lanes of HCT-15, HT-29, and SW837 DNAs indicated that this region was not methylated in these cell lines. These results confirmed that fragment A was unmethylated in 75% (three of four) of colon cancer cell lines previously analyzed by methylation-sensitive AP-PCR. The design of the primer used for AP-PCR made it possible to amplify genomic DNA fragments that could produce PCR products with flanking CCGG sequences defined by the primer annealing sites. Southern analysis of both primary tissues and cell lines revealed the presence of a band in the Rsal + MspI-digested lanes that was approximately 900 bp in size. The size of this fragment on the Southern blot matched the size of the PCR product that was produced by methylation-sensitive AP-PCR analysis of these DNAs. The results of the Southern analysis of both primary tissues and cell lines indicated that there must be two CCGG sites at the termini of these fragments that were hypomethylated in the genomic DNAs examined by methylation-sensitive AP-PCR. The clear differences observed by Southern analysis using fragment A as a probe confirmed that methylation-sensitive AP-PCR is capable of identifying methylation changes at random but specific sites in genomic DNA.

Fig. 3. Southern blot analysis of primary colon tissues and colon cancer cell line DNAs using isolated fragment A as a probe. A, hybridization patterns indicating hypomethylation of fragment A in tumors compared to normal controls. B, hybridization patterns indicating lack of methylation of fragment A in three of four colon cancer cell lines previously analyzed by methylation-sensitive AP-PCR. Ten μg each of matched normal and tumor colon DNA were digested separately with either Rsal (R), Rsal + HpaII (H), or Rsal + MspI (M), electrophoresed, blotted, and probed with plasmid cloned bands to confirm methylation changes.
The remaining 4 fragments (B–E) identified by AP-PCR were also used as probes for Southern analysis. Fragments B and C produced high background on Southern blots, which could not be clearly evaluated perhaps due to the presence of sequences associated with repetitive elements or pseudogenes. Blots of colon cancer cell line DNAs probed with fragment D produced methylation patterns consistent with those observed by AP-PCR, and fragment E was not used as a probe because it was known to contain homology to Alu repetitive sequences. A total of three other DNA fragments that were isolated from AP-PCR polyacrylamide gels but showed no methylation differences between normal and tumor colon tissues and cell lines were also used as probes on Southern blots to verify the methylation patterns identified by AP-PCR. Hypermethylation of HpaII sites within the novel CpG island in tumors previously detected by AP-PCR was also confirmed in colon tissues by using the cloned DNA fragment as a probe (data not shown).

Discussion

Digestion of genomic DNA with methylation-sensitive restriction enzymes followed by low-stringency PCRs allowed us to identify bands that were differentially methylated in normal and tumor colon DNAs and cell lines. The random association of primers with genomic DNA at low annealing temperatures would be expected to generate multiple PCR fragments. If two primer annealing sites flanked at least one unmethylated HpaII restriction site, then no PCR product would be expected to be generated due to HpaII cutting. The presence of methylation at HpaII sites located between primers during AP-PCR would allow for the amplification of certain regions of DNA. The MLG2 primer was designed with a CCGG sequence at its 3′ end to increase the probability of primer annealing to HpaII and MspI restriction sites under low-stringency PCR conditions. This primer design increases the potential number of methylation sites that can be analyzed by the preferential annealing of primers to methylated CCGG sequences, as demonstrated by Southern analysis of primary tissues and cell lines (Fig. 3). Absence of methylation at CCGG sites to which the primers may anneal would produce no amplification product due to HpaII cutting, whereas primer annealing to these sequences could occur in the presence of methylation because of the inability of HpaII to cut methylated CCGG. Methylation changes identified by this technique are therefore not limited to internal CCGG sites within isolated PCR products, but may also include CCGG sequences located at primer annealing sites. The possible formation of primer dimers when using a primer with a 3′ ending in -CCGG did not inhibit the AP-PCR amplification of a large number of DNA fragments (Fig. 1).

Methylation-sensitive AP-PCR is a simple and rapid method that can be used to screen for methylation changes and to isolate specific fragments of DNA associated with these changes in normal and tumor DNA. Southern analysis can be used to study methylation differences between known genes, but its application for isolating specific and unknown regions of DNA associated with methylation changes is limited. In contrast to Southern blotting, which requires large amounts of DNA (5–10 μg), methylation-sensitive AP-PCR can detect methylation changes in as little as 200 ng of genomic DNA. Other techniques such as conventional PCR-based methylation analysis and genomic sequencing require at least partial knowledge of the DNA sequence being studied. Global levels of methylation can be studied using more labor intensive techniques such as high-performance liquid chromatography analysis and RLGS, but methylation-sensitive AP-PCR can be used to screen for genome-wide methylation changes in a simple and rapid manner. This technique uses restriction enzyme digestion and AP-PCR to identify random but specific methylation changes at multiple sites in genomic DNA.

Reproducibility and artifacts are important considerations when dealing with any PCR-based assay. Because many different DNA fragments may be generated by methylation-sensitive AP-PCR, it was critical to determine which species resulted from true methylation changes. The use of a methylation-sensitive restriction enzyme with an isoschizomer was important for controlling for the presence of PCR fragments unrelated to methylation changes. We have also used a double restriction enzyme strategy with Rsal combined with either HpaII or MspI to generate smaller fragments of DNA that potentially contain CCGG sites. Digestion of DNAs with Rsal would also reduce the number of possible regions of DNA that could be amplified by AP-PCR. The use of other restriction enzymes in addition to HpaII and MspI in a double digestion strategy could also be used to detect additional regions of genomic DNA, which may contain differentially methylated sites. Some of the fragments that were amplified by AP-PCR showed no differences between normal and tumor digested DNAs and bands were present in both the normal and tumor HpaII and MspI digestions. These types of fragments clearly did not represent methylation changes, but they did serve as controls for assessing the consistency of PCR reactions between samples. Furthermore, by using fragments identified by methylation-sensitive AP-PCR as probes on Southern blots containing Rsal, Rsal + HpaII, and Rsal + MspI-digested DNA, we confirmed that bands isolated by this method represented true methylation differences between normal and tumor samples.

Methylation-sensitive AP-PCR can be used to identify and isolate regions of genomic DNA that have undergone methylation changes associated with tumorigenesis, much as differential display RT-PCR (29, 30) can be used to identify and isolate differentially expressed sequences. In the present study, we have isolated regions of DNA that showed methylation differences between normal and tumor colon tissues and cell lines. Using various primer combinations and a double restriction enzyme digestion strategy, we have detected regions of DNA hypo- and hypermethylation in the samples analyzed. Previous studies have reported that changes such as global hypomethylation and regional hypermethylation of CpG islands are common events that occur during tumorigenesis (reviewed in Ref. 2). Hypomethylation of genes such as human growth hormone, γ-globin, and α-globin in colon cancers has also been described (28).

In addition to isolating unknown sequences associated with methylation changes in genomic DNA, methylation-sensitive AP-PCR can also be applied to rapidly estimate the variability of methylation at multiple sites between different cell lines or between normal and tumor tissues. We have used methylation-sensitive AP-PCR to screen for methylation differences between normal and tumor DNA. Based on the number of putative methylation sites detected by methylation-sensitive AP-PCR using a single primer in matched sets of normal and tumor colon DNAs, the major differences observed were bands representing putative regions of hypomethylation in tumors compared to normal samples. A total of 44 DNA fragments associated with approximately five different regions of genomic DNA containing methylation sites were identified in a simple and efficient manner that has not been previously described. Furthermore, we have identified and isolated a novel CpG island that was frequently hypermethylated in both bladder and colon tumors by using a dual primer strategy for AP-PCR. Different primer sets were used to demonstrate that other differentially methylated fragments could be identified by this technique. It is interesting to note that regardless of the primer combination used for AP-PCR, identification of hypomethylated regions of DNA was consistently associated with CpG-poor sequences, whereas identification of hypermethylated regions of DNA yielded CpG-rich
METHYLATION-SENSITIVE AP-PCR

sequences. These findings are consistent with the types of methylation changes associated with tumorigenesis as previously described (2). The degree of genome-wide DNA methylation at multiple sites in normal and tumor tissues can be assayed arbitrarily by this technique, and we have shown that it may be possible to examine a number of additional regions of DNA by altering the restriction enzymes used for digestion of DNAs, primer sequences, or PCR conditions. Methylation-sensitive AP-PCR can therefore be used to rapidly screen for methylation differences between genomes and to isolate specific genetic elements that have undergone methylation changes associated with tumorigenesis.

References

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