Identification of Differentially Methylated Sequences in Colorectal Cancer by Methylated CpG Island Amplification

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Abstract

CpG island methylation has been linked to tumor suppressor gene inactivation in neoplasia and may serve as a useful marker to clone novel cancer-related genes. We have developed a novel PCR-based method, methylated CpG island amplification (MCA), which is useful for both methylation analysis and cloning differentially methylated genes. Using restriction enzymes that have differential sensitivity to 5-methyl-cytosine, followed by adaptor ligation and PCR amplification, methylated CpG rich sequences can be preferentially amplified. In a model experiment using a probe from exon 1 of the p16 gene, signal was detected from MCA products of a colorectal cancer cell line but not in normal colon mucosa. To identify novel CpG islands differentially methylated in colorectal cancer, we have applied MCA coupled with representational difference analysis to the colon cancer cell line Caco2 as a tester and normal colon mucosa as a driver. Using this strategy, we isolated 33 differentially methylated DNA sequences, including fragments identical to several known genes (PAX6, Versican, α-tubulin, CSX, OPT, and rRNA gene). The association of hypermethylation of the clones obtained and transcriptional suppression in colorectal cancer was confirmed by examining the Versican gene, which we found to be silenced in methylated cell lines and reactivated by the methylation inhibitor 5-aza-2′-deoxycytidine. We therefore propose that MCA is a useful technique to study methylation and to isolate CpG islands differentially methylated in cancer.

Introduction

In the development of cancer, a series of tumor suppressor genes are inactivated by mutations and chromosomal deletions (1). Aberrant methylation of CpG islands has been shown recently to serve as an alternate way of inactivating such genes in cancer. CpG islands are short sequences rich in the CpG dinucleotide and can be found in the 5′ region of about one-half of all human genes (2). Methylation of cytosine within 5′ CpG islands is associated with loss of gene expression and has been seen in physiological conditions such as X chromosome inactivation (3) and genomic imprinting (4). Aberrant methylation also occurs during aging (5) and carcinogenesis and is linked to transcriptional silencing of multiple genes, including known familial cancer genes (6, 7). This has lead to the hypothesis that novel tumor suppressor genes could be isolated using aberrantly methylated CpG islands as a marker (8). In the past few years, several techniques were developed to detect aberrant methylation in cancer (9–12). Although these techniques are very powerful in detecting methylation differences, they are limited to known genes because they require other information for the design of PCR primers. More recently, other techniques such as restriction landmark genomic scanning and arbitrarily-primed-PCR were used to isolate novel methylated sequences (13–16). However, the number of CpG islands cloned in this way remains relatively limited. To isolate differentially methylated CpG islands in cancer and normal tissues, we have developed a new technique called MCA. MCA allows for the efficient PCR amplification of methylated CpG islands, which can detect methylation of many genes, or to clone CpG islands differentially methylated in cancer. By applying MCA coupled with RDA to colonic tumors, we have isolated 33 sequences hypermethylated in colorectal cancer, including several known genes, and CpG islands that map to areas of loss of heterozygosity in malignancies.

Materials and Methods

Samples and Cell lines. Samples of colon cancer tissues and normal colon mucosa were obtained from The Johns Hopkins Hospital. All patients gave informed consent prior to collection of specimens according to institutional guidelines. All cancer cell lines were obtained from the American Type Culture Collection. Genomic DNA and mRNA were extracted using standard procedures.

MCA. The procedure is outlined in Fig. 1. Five μg of DNA were digested with 100 units of Smal for 6 h (all restriction enzymes were from New England Biolabs). The DNA was then digested with 20 units of Xmal for 16 h. DNA fragments were then precipitated with ethanol. RXMA and RMCA PCR adaptors were prepared by incubation of the oligonucleotides RXMA24 (5′-ACGACTTCTCCAGCCTTCACCCGAC-3′) and RMCA12 (5′-CCGGTTCGGTG-3′) or RMCA24 (5′-CACCAGCATCCAGGGTCTC-3′) and RMCA12 (5′-CCGGGCAGAAAG-3′) at 65°C for 2 min, followed by cooling to room temperature. DNA (0.5 μg) was ligated to 0.5 nmol of RXMA or RMCA adaptor using T4 DNA ligase (New England Biolabs). PCR was performed using 3 μl of each of the ligation mix as a template in a 100-μl volume containing 100 pmol of RXMA24 or RMCA24 primer, 5 units of Taq DNA polymerase (Life Technologies, Inc.), 4 mM MgCl2, 16 mM of NH4(SO4)2, 10 μg/ml of BSA, and 5% v/v DMSO. The reaction mixture was incubated at 72°C at 5 min and at 95°C for 3 min. Samples were then subjected to 25 cycles of amplification consisting of 1 min at 95°C and 3 min either at 72°C or 77°C in a thermal cycler (Hybaid, Inc.). The final extension time was 10 min.

Detection of Aberrant Methylation Using MCA. MCA products from normal colon mucosa and corresponding cancer tissues were prepared as described above. One μg of MCA products was resuspended in 4 μl of TE [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)], mixed with 2 μl of 20× SSC, and 1 μl aliquot of this mix was blotted onto nylon membranes (Nunc) using a 96-well replication system (Nunc). The membranes were baked at 80°C and UV cross-linked for 2 min. Each sample was blotted in duplicate. Each filter included mixtures of a positive control (Caco2) and a negative control (normal colon mucosa from an 18-year-old individual) as shown in Fig. 1B. The hybridization probes (p16 exon 1; Ref. 17) or MINT clones were labeled with 32P by random priming, and the filters were hybridized for 12–16 h, washed, exposed to a phosphor screen for 24–72 h, and developed using a Phos-
Fig. 1. MCA. A, schematic diagram of MCA. A hypothetical fragment of genomic DNA is represented by a solid line, with seven Smal sites depicted by tick marks. m, methylated Smal sites. Fragments B and D are CpG islands. B is methylated in both normal (right) and cancer (left), whereas D is differentially methylated in cancer. For MCA, unmethylated Smal sites are eliminated by digestion with Smal (which is methylation sensitive and does not cut when its recognition sequence CCCGGG contains a methylated CpG), whereas Smal I sites are eliminated by digestion with SmaI, which digests methylated CCCGGG sites, leaving a CCGG overhang (sticky ends). Adaptors are ligated to these sticky ends, and PCR is performed to amplify the methylated sequences. The MCA amplicons can be used directly in a dot blot analysis to study the methylation status of any gene for which a probe is available (left). Alternatively, MCA products can be used to clone differentially methylated sequences by RDA (right). B, semiquantitative detection of p16 methylation using MCA. DNA from the Caco2 cell line and normal colon were mixed in varying proportions prior to MCA, and p16 methylation was analyzed by dot blot analysis. The percentage refers to the relative proportion of Caco2 DNA. 0.1 μg of the MCA products were blotted on a nylon membrane and probed with a labeled p16 exon 1 probe. Each sample was blotted in duplicate. C, examples of dot blot analysis using clones derived from MCA/RDA. The first two clones hybridized to both Caco2 and normal colon MCA products and are not, therefore, differentially methylated. All others hybridize only to Caco2 and are differentially methylated. Lane N: DNA from the normal colon of an 18-year-old individual. D, Southern blot analysis of colorectal cancer and adenomas using MINT3 as a probe. Shown are nine pairs of colorectal tumors (T) and adjacent normal mucosa (N). Digests were carried out with Eagl (methylation-sensitive) and HindIII (flank). Left, DNA sizes. The 4 kb represents methylation of the MINT3 Eagl site and is found in six of nine tumors.

**Southern Blot Analysis.** Five μg of DNA were digested with 20–100 units of restriction enzymes as specified by the manufacturer (New England Biolabs). DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane (Zeta-probe; Bio-Rad). Filters were hybridized with 32P-labeled probes and washed at 65°C with 2× SSC, 0.1% SDS for 10 min twice and 0.1× SSC, 0.1% SDS for 20 min. Filters were then exposed to a phosphor screen for 24–72 h and analyzed using a PhosphorImager (Molecular Dynamics). The intensity of each signal was calculated using Image Quant software, and methylation levels were determined relative to the control samples.

**RDA.** RDA was performed essentially as reported previously (18) with the following modifications. For the first and second rounds of competitive hybridization, 500 and 100 ng of ligation mix were used, respectively. To eliminate the digested adaptor, a cDNA spun column (Amersham) was used instead of excising from the agarose gel. Primers used for the first and second rounds of RDA were as follows: JXMA24 (5'-AGGCCATCGACTCATCGATGACCC-3'), JXMA12 (5'-CCGGGTGACCG-3'), JMAC24 (5'-GT- GAGGTCCGATCTGTGTTGCTC-3'), JMAC12 (5'-CCGGAGGCACCGC-3'), NXMA24 (5'-AGGCACTTGTCTGCTGAGTGCAC-3'), NXMA12 (5'-CCGGGTACTCGA-3'), NMCA24 (5'-GTGACCGGACTACGGG-3'), and NMCA12 (5'-CCGGGTGACCCCG-3'). After the second round of competitive hybridization, PCR products were digested with XmaI. The J adaptor was eliminated by column filtration. The PCR products were then subcloned into pBluescript SK+ (Stratagene). To screen for inserts, a total of 396 clones were cultured overnight in LB medium with ampicillin, and 3 μl of the culture was directly used as template for a PCR reaction. Each clone was amplified with T3 (5'-AATTAACCCCTACAAAGGGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3') primers, blotted onto nylon membranes, and screened for cross hybridization with 32P-labeled inserts. The clones differentially hybridizing to tester and driver MCA products were further characterized by Southern blot analysis and DNA sequencing. All sequences were deposited in GenBank.
Results

Identification of Differentially Methylated CpG islands in Colorectal Cancer by MCA/RDA. To identify novel CpG islands aberrantly methylated in colorectal cancer, we used RDA, a technique that was developed to clone small differences between genomes (18). RDA is a subtraction technique that relies on hybridizing the two genomes of interest (tester and driver), followed by PCR amplification of tester sequences that did not hybridize with driver DNA. In this study, MCA was used to enrich for hypermethylated CpG islands, and RDA was used to identify those that are exclusively methylated in cancer. RDA was performed on MCA amplicons from the colon cancer cell line Caco2 as a tester, and a mixture of DNA from the normal colon mucosa of five different men (to avoid cloning polymorphic Smal sites or inactive and methylated X chromosome genes from women) as a driver. Two separate experiments were conducted, one using a lower annealing temperature (72°C), and the other using a higher annealing temperature (77°C) and more GC-rich primers to amplify GC-rich sequences. After two rounds of RDA, the PCR products were cloned, and colonies containing inserts were identified by PCR. On the basis of initial experiments, we expected most of the recovered clones to contain Alu-repetitive sequences, which are GC rich and hypermethylated (20). All clones were therefore probed with an Alu fragment, and only nonhybridizing clones were analyzed further. Of 160 non-Alu clones, 46 were independent clones, and 33 of these (MINT1–33) appeared to be differentially methylated in Caco2 cells by comparing hybridization to MCA products from Caco2 and normal colon (Fig. 1C). Nineteen of the clones (MINT1–19) were obtained using the lower annealing temperature, and 14 (MINT 20–33) were obtained using the higher temperature.

To confirm the accuracy of MCA, differential methylation was confirmed by Southern blot analysis in all cases (Fig. 1D and data not shown). All of the 33 clones were hypermethylated in Caco2 compared with normal colon mucosa. Of these 33, one clone (MINT13) detected highly repeated sequences, and two clones (MINT18 and MINT28) appeared to correspond to mildly repeated gene families (data not shown). All others appeared to detect single-copy DNA fragments.

By DNA sequencing, we found that 29 clones had a GC content >50% and satisfied the minimal criteria for CpG islands (200 bp, GC content >50%, CpG/GpC >0.5; Ref. 21). As might be expected, clones obtained with the higher annealing temperature and more GC-rich primers had a relatively higher GC content (Table 1). The size of each clone, percentage of GC nucleotide, CpG/GpC, sequence homology, chromosomal location, and GenBank accession numbers are summarized in Table 1. MINT5, MINT8, MINT11, MINT14, and MINT16 contained GC-rich regions only in one end of the clones, and these may have been recovered from the edge of CpG islands.

By DNA homology search using the BLAST program, 6 clones were identical to human gene sequences, 4 clones were identical to CpG islands randomly sequenced from a CpG island library (22), 1 was identical to an EST, 3 clones were identical to high-throughput genomic sequences deposited in GenBank, 3 had significant homology to other genes, and the other 19 had no significant match in the database; MINT8 was identical to PAX6 enhancer, MINT11 was identical to exon 1 and intron 1 of the human Versican gene and corresponded to the 3′ edge of a promoter-associated CpG island; MINT14 was identical to exon 1 of the human α-tubulin gene and was also the 3′ edge of the CpG island; MINT 24 corresponded to the 3′ noncoding region of the human homeobox gene CSX; MINT21 had a
region with 94% homology at the nucleotide level to exon 2 of the mouse \textit{OPT} gene and probably represents the human homologue of this gene; and MINT28 was homologous to ribosomal gene sequences (Fig. 2; summarized in Table 1). To examine the presence of potential promoter sequences in these clones, promoter prediction was performed using several computer programs. Twenty of the 33 clones were predicted as promoters using the NNPP program, and six were predicted as promoters by using the TSSG program.

The chromosomal position of most of the unknown clones was determined using a somatic cell hybrid panel and a radiation hybrid panel (Table 1). Of note, MINT3 and MINT9 mapped to chromosome 1p35–36, MINT13 mapped to 7q31, MINT24 mapped to 3p25–26, MINT25 mapped to 22q11–ter, and MINT31 mapped to 17q21. All of these chromosomal segments are areas that are frequently deleted in various tumors (23).

\textbf{Silencing of the Versican Gene in Colorectal Cancer.} To determine whether some of these clones truly represented genes silenced by methylation, we examined the \textit{Versican} gene in more detail. \textit{Versican} is a secreted glycoprotein that appears to be regulated by the \textit{RB1} tumor suppressor gene (24). MINT11 corresponds to part of exon 1 and part of intron 1 of the \textit{Versican} gene (Fig. 2). Hypermethylation of the two \textit{Smal} sites in exon 1 and intron 1 in colon cancer cell lines was confirmed by both Southern blot analysis and MCA (data not shown). We hypothesized that this methylation was representative of the entire CpG island, including the proximal promoter. To address this issue, we used PCR of bisulfite-treated DNA using primers designed to amplify the region around the transcription start site of this gene. The PCR product was then digested with restriction enzymes that distinguish methylated from unmethylated DNA (Fig. 3A). The \textit{Versican} promoter was found to be completely methylated in the colon cancer cell lines DLD1, LOVO, SW48, and SW837 and partially methylated in HCT116 and HT29 (Fig. 3B). In primary colon tumors, \textit{Versican} was hypermethylated in 17 of 25 cases (68%; Fig. 3B). Interestingly, some methylation of the \textit{Versican} promoter was also found in normal tissues, albeit at lower levels when compared with tumors. The level of methylation in normal colon mucosa increased with age of the patient (Fig. 3, B and C), from an average of

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Clone & Size (bp) & %GC & CpG/GpC & CpG island\textsuperscript{a} & Blast homology & Chromosome map & Genbank accession no. \\
\hline
MINT1 & 528 & 56 & 0.6 & Yes & None & 5q13-14 & AF135501 \\
MINT2 & 562 & 50 & 0.8 & Yes & None & 2p22-21 & AF135502 \\
MINT3 & 563 & 55 & 1 & Yes & Human EST & 1p34-35 & AF135503 \\
MINT4 & 481 & 60 & 0.8 & Yes & None & 15q25-26 & AF135504 \\
MINT5 & 852 & 46 & 0.5 & Yes\textsuperscript{b} & Human CpG clone\textsuperscript{c} & 14q11-22 & AF135505 \\
MINT6 & 400 & 59 & 0.6 & Yes & None & 12q14-15 & AF135506 \\
MINT7 & 481 & 49 & 0.9 & Yes & Human genomic DNA\textsuperscript{d} & 6p21-22 & AF135507 \\
MINT8 & 617 & 46 & 0.5 & Yes\textsuperscript{b} & \textit{PAX6} enhancer & 11p13 & AF135508 \\
MINT9 & 605 & 54 & 0.3 & No & None & 1p34-35 & AF135509 \\
MINT10 & 608 & 49 & 0.6 & Yes & None & 9q34–ter & AF135510 \\
MINT11 & 636 & 48 & 0.5 & Yes\textsuperscript{b} & Versican & 5q12-13 & AF135511 \\
MINT12 & 552 & 49 & 0.6 & Yes & Human CpG clone & 7q31-32 & AF135512 \\
MINT13 & 313 & 60 & 0.9 & Yes & \textit{LINE1} & ND\textsuperscript{e} & AF135513 \\
MINT14 & 620 & 54 & 0.4 & Yes\textsuperscript{b} & None & 10p13-15 & AF135514 \\
MINT15 & 641 & 53 & 0.7 & Yes & None & 11p12-13 & AF135515 \\
MINT16 & 664 & 62 & 0.5 & Yes\textsuperscript{b} & \textit{a-Tubulin} & 2q & AF135516 \\
MINT17 & 490 & 54 & 0.7 & Yes & None & 6 & AF135517 \\
MINT18 & 435 & 58 & 0.1 & No & None & ND & AF135518 \\
MINT19 & 443 & 55 & 0.2 & No & None & ND & AF135519 \\
MINT20 & 510 & 67 & 0.8 & Yes & Mouse \textit{OTP} & ND & AF135520 \\
MINT21 & 410 & 62 & 0.4 & No & Human genomic DNA & 22q13 & AF135521 \\
MINT22 & 438 & 60 & 0.9 & Yes & None & 10p12 & AF135522 \\
MINT23 & 414 & 64 & 0.9 & Yes & \textit{CSX} & 5q34-35 & AF135523 \\
MINT24 & 523 & 67 & 0.7 & Yes & Human EST & 3p25-26 & AF135524 \\
MINT25 & 339 & 60 & 0.7 & Yes & Human genomic DNA & 22q11 & AF135525 \\
MINT26 & 591 & 58 & 0.8 & Yes & Human CpG clone & 7q11 & AF135526 \\
MINT27 & 242 & 74 & 0.7 & Yes & None & ND & AF135527 \\
MINT28 & 463 & 58 & 1 & Yes & Ribosomal RNA gene & ND & AF135528 \\
MINT29 & 429 & 60 & 0.7 & Yes & Human CpG clone & 7q11 & AF135529 \\
MINT30 & 536 & 65 & 0.5 & Yes & None & 20q11 & AF135530 \\
MINT31 & 673 & 65 & 0.8 & Yes & Human genomic DNA & 17q21 & AF135531 \\
MINT32 & 464 & 66 & 1 & Yes & None & 20q13 & AF135532 \\
MINT33 & 139 & 65 & 0.8 & Yes & None & ND & AF135533 \\
\hline
\end{tabular}
\caption{Summary of the 33 differentially methylated clones isolated by MCA-RDA}
\end{table}
METHYLATED CpG ISLAND AMPLIFICATION

Fig. 3. Hypermethylation and loss of expression of the Versican gene in colorectal cancer. A, map of the Versican gene first exon (filled box) and flanking regions. Top, CpG sites. Arrows, location of the primers used for bisulfite-PCR. Methylated alleles are digested to 162 and 37 bp by the restriction enzyme TaqI. Unmethylated alleles remain undigested because of the absence of this site after bisulfite conversion of unmethylated cytosine. B, Versican methylation in colorectal cancer and normal colon mucosa. Bisulfite-treated DNA was amplified using primers V1 and V2. The PCR product (199 bp) was digested with TaqI and electrophoresed in a 3% agarose gel. TaqI cleaves only the methylated allele, yielding bands at 162 and 37 bp (the 37 bp is not visible by agarose gel electrophoresis). Hypermethylation of the Versican gene was detected in most of the colorectal cancer cell lines tested (top) and 70% of primary colorectal cancer (T1, T2, and T4–6; middle panel). N, normal colon; T, colon tumor. Methylation of Versican was also detectable in normal colon mucosa and progressively increased with age (bottom panel, all samples are from normal colon mucosa; age of the patient is indicated on top of each lane). Percentage Versican methylation (shown at the bottom of each lane) was determined by comparing the intensity of the bands derived from the methylated (162 bp) and unmethylated (199 bp) alleles. C, age-related methylation of Versican in colonic tissues. Columns, average percentages of methylation for each decade; bars, SEM. The number of samples examined for each decade is 4, 6, 3, 6, 4, 9, 10, and 4, respectively. D, expression of Versican (V) in colonic tissues. Versican expression was determined by RT-PCR in normal colon (left), hemimethylated cell lines (HCT116 and HT29), highly methylated cell lines (LOVO, SW48, SW837, RKO, CEM, RAJI, DLD1, Caco2, and SW480), and colorectal cancer cell lines treated with the methylation inhibitor 5-aza-2'-deoxycytidine (tr). All reactions were controlled by RT (−) samples (data not shown). The cDNAs were also amplified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (G) to verify RNA integrity. Sizes of the PCR products are 292 bp for Versican and 306 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Note that all of the highly methylated cell lines have very low levels of expression (compared with normal colon), and that this expression is significantly increased by treatment with the methylation inhibitor (right).

Discussion

MCA is a novel PCR-based technique that allows for the rapid enrichment of hypermethylated CG-rich sequences, with a high representation of methylated CpG islands. This technique can have several potential applications. MCA can be useful for the determination of the methylation status of a large number of samples at multiple loci relatively rapidly. By optimizing the PCR conditions, it should be readily adaptable to the study of the methylation status of any gene that has two closely spaced Smal sites. As shown here, there is a very high concordance rate between MCA and other methods for the detection of hypermethylation, such as Southern blot analysis and bisulfite-based methods. However, compared with other methods, MCA has some disadvantages in that: (a) it requires good quality DNA, excluding the study of paraffin-embedded samples; (b) it examines only a limited number of CpG sites within a CpG island; and (c) it is sensitive to incomplete digestion using the methylation-sensitive enzyme Smal. Nevertheless, many steps in MCA are amenable to automation and, by allowing for the examination of multiple genes relatively quickly, it may have important applications in population-based studies of CpG island methylation.

An important application of MCA is in the discovery of a novel gene hypermethylated in cancer. As demonstrated here, MCA coupled with RDA is a rapid and powerful technology for this purpose and compares favorably with other described techniques (13–16). In ad-

6.9% in patients between 20 and 30 years of age, to an average of 28.9% in patients >80. A linear regression analysis revealed a significant association between age and Versican promoter methylation (r = 0.7, P < 0.000001). Using RT-PCR, we next examined the expression of Versican in normal colon mucosa and colorectal cancer cell lines. As shown in Fig. 3D, Versican is expressed in normal colon epithelium but is markedly down-regulated or absent in methylated colon cancer cell lines. Expression of Versican in all of these cell lines was easily restored after treatment with the demethylating agent, 5-aza-2'-deoxycytidine (Fig. 3D). These data suggest that Versican becomes methylated in normal colon in an age-dependent manner, and that this leads to hypermethylation and loss of expression in most colorectal tumors.
dition to the identification of genes hypermethylated in cancer, MCA could potentially be used to discover novel imprinted genes using parthenogenetic DNA, as well as novel X chromosome genes.

Using MCA/RDA, 33 differentially methylated clones were identified and characterized in detail. By sequencing, we found that 29 of the 33 clones satisfy the criteria of CpG islands, demonstrating that MCA can represent CpG islands specifically. Of these 29 clones, 6 were already known genes (PAX6, Versican, α-tubulin, CSX, OPT homologue, and RNA gene).

Of the known genes recovered in this study, Versican is most interesting in that this proteoglycan is an RBP-inducible gene (24), suggesting that down-regulation of this gene product may have an important role in colorectal carcinogenesis, where RB mutations are rare. Although the relation between Versican and cancer was first described for hypomethylation of this gene involving a nonpromoter region (25), our data clearly show that aberrant methylation of the Versican gene promoter is correlated with silencing of this gene. Here, we also show that promoter methylation of Versican is associated with aging in normal colon mucosa. This is similar to other genes reported to be methylated as a function of age in normal colon, including ER (5), IGF2 (26), MyoD, and N33 (27). Although the functional significance of age-related methylation is still not clear, we have suggested that it constitutes a type of field defect in the colon, which partially explains the dramatic age-related increase in colorectal cancer incidence (5).

Methylation of the CSX and OPT genes do not coincide with their 5’ end and is therefore not expected to silence these genes. It is possible, however, that these CpG islands are associated with alternate transcripts of the genes or with other nearby genes, which would then be silenced by methylation (28). Nevertheless, these data confirm previous studies showing that hypermethylation in cancer does not always involve promoter-associated CpG islands (29). PAX6 is a gene involved in eye development that, interestingly, was also identified as differentially methylated in cancer using arbitrarily-primed-PCR (29).

In that study, the CpG island recovered was in the coding region of the gene, whereas MINT8 corresponds to a PAX6 enhancer present in the 5’ region of the gene. Methylation of the PAX6 enhancer is likely to influence its transcription. However, it is not known whether this gene is expressed in normal colonic tissues. Finally, methylation of ribosomal genes has been seen previously in aging tissues (30) and therefore is not surprising to find in cancers.

The significance of methylation of the other clones obtained in this report is not clear. The methylation of some of these may simply reflect the global redistribution of 5-methylcytosine during cancer development (6, 7) with little functional significance. However, because some of the clones recovered are in the exon 1 region of expressed genes, identification of new tumor suppressor genes might be facilitated by using MCA/RDA clones as probes for screening cDNA libraries. Indeed, on the basis of their chromosome location, several clones map to chromosomal regions thought to harbor tumor suppressor genes because they are highly deleted in various tumors (e.g., chromosomes 1p35, 3p25–26, 7q31, 17q21, and 22q11–ter).

In conclusion, we have developed MCA, a novel method to selectively amplify methylated CpG islands. Using MCA coupled with RDA, 33 clones differentially methylated in colorectal cancer were isolated, including several known genes. These clones will be useful markers to identify novel genes silenced by hypermethylation in cancer and may also be useful markers for early detection and prediction of prognosis in colorectal cancer.

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