Discovery of Epigenetically Silenced Genes by Methylated DNA Immunoprecipitation in Colon Cancer Cells

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Abstract

CpG island promoter hypermethylation of tumor suppressor genes is a common hallmark of human cancer, and new large-scale epigenomic technologies might be useful in our attempts to define the complete DNA hypermethyome of tumor cells. Here, we report a functional search for hypermethylated CpG islands using the colorectal cancer cell line HCT-116, in which two major DNA methyltransferases, DNMT1 and DNMT3b, have been genetically disrupted (DKO cells). Using methylated DNA immunoprecipitation methodology in conjunction with promoter microarray analyses, we found that DKO cells experience a significant loss of hypermethylated CpG islands. Further characterization of these candidate sequences shows CpG island promoter hypermethylation and silencing of genes with potentially important roles in tumorigenesis, such as the Ras guanine nucleotide-releasing factor (RasGRF2), the apoptosis-associated basic helix-loop transcription factor (BHLHB9), and the homeobox gene (HOXD1). Hypermethylation of these genes occurs in premalignant lesions and accumulates during tumorigenesis. Thus, our results show the usefulness of DNMT genetic disruption strategies combined with methylated DNA immunoprecipitation in searching for unknown hypermethylated candidate genes in human cancer that might aid our understanding of the biology of the disease and be of potential translational use. [Cancer Res 2007;67(24):11481–6]

Introduction

Inactivation of tumor suppressor genes in human cancer occurs very often by hypermethylation of the CpG islands located in the promoter regions of these genes (1–3). Global cytosine methylation patterns in mammals seem to be established by a complex interplay of at least three independently encoded DNA methyltransferases (DNMT): DNMT1, DNMT3a, and DNMT3b (1–3). Homologous recombination has been used in the colorectal cancer cell line HCT-116 to disrupt DNMT1 or/and DNMT3b (4, 5). Single DNMT knockouts had minor changes in DNA methylation (4, 5) that, in part, might be associated with the presence of recently identified alternative transcripts arising from the DNMT1 gene (6). However, most important, the HCT-116 double-knockout cells for DNMT1 and DNMT3b (DKO cells; ref. 5) showed a minimal DNA methyltransferase activity, a 95% reduction in 5-methylcytosine content, demethylation of repeated sequences, loss of imprinting at the insulin-like growth factor II locus, and abrogation of the methylation-mediated silencing of the tumor suppressor genes p16INK4a and TIMP-3 (5).

Among others, the candidate gene and genomic and pharmacologic approaches have all been used in the search for new genes that undergo methylation-associated inactivation in cancer cells (1–3), but the DNMT genetic avenue has not yet been fully explored. We wondered about the extent of CpG island hypomethylation events in the DKO cells and whether these cells could be used to find new genes with hypermethylation-associated inactivation in human cancer. In our preliminary genomic screenings, we observed CpG island hypomethylation events in putative tumor suppressor genes (7). The recent introduction of methylated DNA immunoprecipitation (MeDIP) technology, combined with comprehensive gene promoter arrays (8–10), prompted us to reinvestigate the DKO cells using this new epigenomic tool. Our results show that cancer cells lacking DNMT1 and DNMT3b undergo significant CpG island hypomethylation events that identify new putative tumor suppressor genes undergoing methylation-associated silencing in human cancer. These data contribute to a more complete map of the DNA hypermethylome of malignant cells and provide new hypermethylated markers for putative translational use in patients with colorectal cancer.

Materials and Methods

Human cancer cell lines and primary tumor samples. HCT-116 colon cancer cells and double DNMT1−/−/DNMT3b−/− (DKO) cells were grown as previously described (5). HCT-116 cells were treated with 5-aza-2-deoxycytidine (1 μmol/L) for 72 h. HCT116 and DKO cells were a generous gift from Dr. Bert Vogelstein (Johns Hopkins Kimmel Comprehensive Cancer Center, Baltimore, MD). All the other human colon cancer cell lines (n = 7) were obtained from the American Type Culture Collection. Tissue samples of primary colorectal tumors (n = 72), adenomas (n = 34), and normal colon (n = 10) were all obtained at the time of the clinically indicated procedures.

MeDIP and microarray hybridization. The MeDIP assay was developed as previously described (8). Four micrograms of genomic DNA extracted from HCT116 and DKO nuclei were sonicated to produce random fragments ranging in size from 300 to 600 bp. We denatured the DNA for 10 min at 95 °C and immunoprecipitated it overnight at 4 °C with 10 μl of monoclonal antibody against 5-methylcytidine (1 mg/mL; Eurogentec). Real-time PCR on MeDIP samples was carried out using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Immunoprecipitated methylated DNA was labeled with Cy5 fluorophore and the input genomic DNA was labeled with Cy3 fluorophore. Labeled DNA from the enriched and the input pools were combined (1–2 μg) and hybridized to the Human Proximal Promoter Array 44K (Agilent Technologies), which contained 60-mer oligonucleotide probes covering the region from −1 to +0.3 kb relative to the transcript start sites for 17,917 annotated human genes. Arrays were then washed and scanned with an Agilent DNA microarray scanner. Data normalization and analysis was developed as described in the Supplementary Methods. To obtain more information about the biological features of the hypermethylated candidate genes and to check the biological coherence of the results obtained, we used the Fatigo program (11).
DNA methylation analysis of candidate genes. The CpG Island Searcher Program (12) was used to determine which genes had a CpG island in their 5'-ends. DNA methylation status was established by PCR analysis of bisulfite-modified genomic DNA. Using two procedures, this induces the chemical conversion of unmethylated, but not methylated, cytosine to uracil. First, methylation status was analyzed by bisulfite genomic sequencing of both strands of the corresponding CpG islands. The second procedure used methylation-specific PCR involving primers specific to either the methylated or modified unmethylated DNA (Supplementary Table S1).

Semiquantitative reverse transcription PCR expression analysis. We reverse-transcribed total RNA (2 μg) treated with DNase I (Ambion) using oligo(dT)12 to 18 primer with SuperScript II reverse transcriptase (Life Technologies). We used 100 ng of cDNA for PCR amplification, and we amplified all of the genes with multiple cycle numbers (20–35 cycles) to determine the appropriate conditions for obtaining semiquantitative differences in their expression levels. Reverse transcription PCR primers were designed between different exons to avoid any amplification of DNA (Supplementary Table S1).

Results and Discussion

The MeDIP microarray profile of wild-type HCT-116 colon cancer cells and DNA methyltransferase-deficient cells (DKO). MeDIP has been used in conjunction with genomic microarrays in transformed and normal cells to outline the DNA methylation differences associated with tumorigenesis in several recent, promising studies (8–10). We have applied the MeDIP approach to a 44K human proximal promoter array to evaluate the CpG hypomethylation changes in the DNMT1/DNMT3b double knock-out HCT-116 cells (DKO) in relation to the wild-type HCT-116 to reveal newly hypermethylated genes in colorectal tumors. The methodology is summarized in Fig. 1A. The entire set of raw and preprocessed microarray data is deposited in the National Center for Biotechnology Information Gene Expression Omnibus1 (accession number GSE9267) and is also accessible at our ftp repository.2 To test the specificity and efficiency of MeDIP, we compared the relative enrichment of known methylated and unmethylated genomic sequences using real-time PCR. MeDIP-enriched methylated DNA, as exemplified by the cancer-specific promoter hypermethylation of the retinoic acid receptor B2 (RARB2; refs. 1–3) and the imprinting control regions of H19 and GPR109A (refs. 8, 13; Fig. 1B), in comparison with unmethylated CpG sequences, such as the histone H3B promoter (ref. 10; Fig. 1B). Most importantly, DKO cells showed markedly depleted MeDIP enrichment in comparison with HCT-116 cells for the methylated DNA sequences, such as RARB2, H19, and GPR109A (Fig. 1B). Only 28 promoters of 17,917 annotated human genes (0.15%) retained MeDIP enrichment in DKO cells in comparison with wild-type HCT-116 cells (Fig. 1C; Supplementary Table S3). Using the CpG Island Searcher Program (11), we estimated that 103 (82%) of these candidate genes had a CpG island in their 5'-ends (Fig. 1C). Gene ontology analyses of these 103 candidate hypermethylated genes revealed a broad representation of all the common hallmark pathways of cancer cells (14), such as DNA repair, and cell death, although transcriptional regulators were clearly overrepresented (Fig. 1D). This latter observation is of particular interest because many hypermethylated promoter CpG islands in cancer cells correspond to genes with a critical role in the regulation of transcription, such as SFRPs-1, GATAs, HIC-1, DKK-1, and TWIST2 (1–3, 15).

To gain further knowledge of the different DNA methylation patterns of the genes enriched after MeDIP in HCT-116 and DKO cells, we randomly selected nine of these gene-associated CpG islands for further characterization by bisulfite genomic sequencing of multiple clones. The genes selected were the Ras protein–specific guanine nucleotide-releasing factor 2 (RASGRF2), the sodium channel non–voltage-gated 1β (SCNN1B), the homeobox D1 (HOXD1), the Polo-like kinase 2 (PLK2), the basic helix-loop-helix domain containing class B9 (BHLB9), ubiquitin-conjugating enzyme E2 variant 2 (UBE2V2), the ElaC homologue 2 (ELAC2), the B3-interacting domain death agonist (BID), and the PH domain and leucine-rich-repeat protein phosphatase 1 (PLEKHE1). We observed that 78% (seven of nine) of these CpG islands were densely methylated in HCT-116 cells and fully unmethylated in DKO cells (Figs. 1C and 2A). The genes were RASGRF2, SCNN1B, HOXD1, PLK2, BHLB9, and UBE2V2. In the three remaining genes, ELAC2, BID, and PLEKHE1, the 5’-associated CpG islands were unmethylated in both HCT-116 and DKO cells (Supplementary Fig. S1). We wanted to focus on cancer-specific DNA hypermethylation, and so we analyzed the DNA methylation status of the six CpG islands found to be hypermethylated in HCT-116 cells by bisulfite genomic sequencing in a collection of normal colon tissues (n = 10). We observed that 67% (four of six) of these CpG islands were always unmethylated in the normal tissues, and thus, their methylation was cancer-specific: this was the case for RASGRF2, SCNN1B, HOXD1, and PLK2 (Fig. 2A and B).

For the two remaining genes, the CpG island UBE2V2 was consistently methylated in all normal colon samples studied (data not shown), but the case of the second gene, BHLB9, was particularly interesting due to its chromosomal location on the X-chromosome (Xq23), that, in females, is randomly inactivated by DNA methylation in one copy. Thus, the normal tissues in which we found BHLB9 CpG island hypermethylation were all from female donors whereas normal tissues from male donors were always unmethylated (Supplementary Fig. S2). Most importantly, because the HCT-116 cancer cells originated from a male (16), and thus had not undergone X-inactivation and its associated BHLB9 CpG island hypermethylation, the presence of BHLB9 hypermethylation in the HCT-116 malignant cells could be considered a cancer-specific hypermethylation event, similar to those described for the other four newly identified candidates (RASGRF2, SCNN1B, HOXD1, and PLK2).

CpG island hypermethylation in the newly identified candidate genes is associated with transcriptional inactivation. It is critical to establish the effect of the detected 5′-end CpG island hypermethylation events on the expression of the contiguous genes. The presence of PLK2 hypermethylation-associated silencing has recently been described (17), and for this reason, we undertook no further studies. For the four remaining hypermethylated cancer-specific genes (RASGRF2, SCNN1B, HOXD1, and BHLB9), we addressed the association between DNA methylation and expression analyzed by semiquantitative reverse transcription PCR. For all four genes, hypermethylated HCT-116 cells showed loss of expression of their respective transcripts (Fig. 2C).

2 http://www.cnics.es/es/index.asp
Figure 1. Unmasking of epigenetically silenced genes using MeDIP. A, explanatory illustration of the MeDIP approach used. DNA methylation levels are calculated as the average of oligonucleotide ratios between immunoprecipitated 5-methylcytosine (IP >5mC) versus input. The confidence of binding calls is represented as a P value. In the graph, the probes marked with a red square (set P < 0.001) were considered to be potentially methylated (Bound). B, validation by real-time PCR of the enriched DNAs obtained from the MeDIP assays. Highly methylated promoters from the imprinted genes H19 and GPR109 and the tumor suppressor gene RARα2 (hypermethylated in HCT-116) were selected as positive controls to measure the enrichment levels obtained after MeDIP. The graph shows a specific and efficient enrichment of methylated DNA over an unmethylated promoter (H3b) used as a negative control. C, schematic strategy used to identify cancer-specific promoter hypermethylation in colon cancer cells using MeDIP. D, gene ontology categories of the 126 hypermethylated candidate genes obtained from the MeDIP approach. Ontology terms (Y-axis), percentage of enrichment (X-axis).
importantly, restoration of expression was observed upon treat-
ment with the demethylating agent 5-aza-2-deoxycytidine and was
also observed in DKO cells (Fig. 2C).

Furthermore, to determine whether these genes display putative
tumor suppressor features, we examined the effect of the ectopic
expression of RASGRF2 in the growth of wild-type HCT-116 cells. In
the colony formation assay, we used G418 selection after
transfecting the HCT-116 cell line with the RASGRF2 gene or the
empty vector (Supplementary Fig. S3). RASGRF2 reexpression
revealed tumor suppressor activity whereby there was a reduction
of 56% in colony formation density with respect to the empty
vector (Supplementary Fig. S3).

The presence of hypermethylation of RASGRF2, SCNN1B, HOXD1,
and BHLB9 was not a unique feature of the HCT-116 colon cancer

![Figure 2](https://cancerres.aacrjournals.org/articles/67/24/11484/)

**Figure 2.** CpG island DNA methylation and expression analyses of the cancer-specific hypermethylated genes found in the MeDIP approach. **A,** bisulfite genomic sequencing analyses of SCNN1B, RASGRF2, BHLHB9, and HOXD1 CpG island methylation status in HCT116, DKO and normal colon. CpG dinucleotides
*short vertical lines*). The transcriptional start site (*long black arrow*) and the location of bisulfite genomic sequencing PCR primers (*white arrows*). Ten single clones are represented for each sample. The presence of a methylated (*black squares*) or unmethylated (*white squares*) cytosine. The four CpG islands are hypermethylated
in HCT-116 cells, but unmethylated in DKO and normal colon. **B,** illustrative methylation-specific PCR analyses for SCNN1B, RASGRF2, BHLHB9, and HOXD1 gene
in human normal colon samples (*NC 1–5*). The presence of a PCR band indicates methylated (*lane M*) or unmethylated (*lane U*) genes. DNA from normal
lymphocytes (*NL*) and *in vitro* methylated DNA (*IVD*) were used as positive and negative controls for methylated DNA, respectively. For BHLHB9, only colon samples
from male donors were used. The four CpG islands were unmethylated in normal colon. **C,** expression analyses for SCNN1B, RASGRF2, BHLHB9, and HOXD1 using
reverse transcription PCR. Hypermethylated HCT-116 cells show loss of expression of the respective transcripts and restoration of expression is observed upon
treatment with the demethylating agent 5-aza-2-deoxycytidine (*DAC*) and in DKO cells. The water reaction and normal colon are shown as negative and positive
controls, respectively.
cell line; upon analyzing a collection of colorectal cancer cell lines (n = 7), we observed the presence of these epigenetic alterations (Fig. 3A; Table 1). The only exception was HOXD1, which was only hypermethylated in HCT-116. Because all cell lines used originated from male colorectal patients, except SW48, the normal X-chromosome–related hypermethylation of BHLB9 in females was not an issue. The association between CpG island hypermethylation of each gene and loss of expression shown above in HCT-116 cells, was also found in this panel of cancer cell lines (Fig. 3B).

**CpG island hypermethylation profile for the newly identified candidate genes in human colorectal tumorigenesis.** The presence of RASGRF2, SCNN1B, HOXD1, and BHLB9 hypermethylation was not an in vitro cell culture phenomenon, but when a

### Table 1. CpG island hypermethylation distribution of the MeDIP-identified candidate genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Colorectal cancer cell lines</th>
<th>Colorectal primary tumors</th>
<th>Adenomas</th>
<th>Normal colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCNN1B</td>
<td>88% (7/8)</td>
<td>20% (11/56)</td>
<td>13% (4/30)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>RASGRF2</td>
<td>38% (3/8)</td>
<td>45% (29/65)</td>
<td>35% (12/34)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>BHLB9*</td>
<td>42% (3/7)</td>
<td>33% (18/54)</td>
<td>33% (9/27)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>HOXD1</td>
<td>13% (1/8)</td>
<td>4% (2/52)</td>
<td>0% (0/33)</td>
<td>0% (0/10)</td>
</tr>
</tbody>
</table>

*For BHLB9, located in the X-chromosome, only samples from male donors were included.
collection of primary tumor samples from colorectal cancer patients was analyzed, it was also observed (Fig. 3C; Table 1). The presence of hypermethylation of any of the described four genes was not associated with any particular clinical stage, age of the patient, or anatomic location in the colon. Furthermore, in 19 colorectal primary tumors in which RNA was available, the promoter CpG island methylation of RASGRF2, SCNN1B, HOXD1, and BHLB9 was associated with the loss of their corresponding transcripts (Fig. 3C).

To determine whether hypermethylation of the described genes might represent an early lesion in colorectal tumorigenesis, we examined the CpG island methylation status of RASGRF2, SCNN1B, HOXD1, and BHLB9 in benign colorectal adenomas, a lesion that is a precursor to invasive colorectal tumors. We observed a hypermethylation frequency similar to that of invasive colon carcinomas (Fig. 3D; Table 1), which is also present in both small (<15 mm) and large adenomas (>15 mm). These findings point to hypermethylation of the identified candidate genes as early events in the pathway to full-blown colorectal tumors.

Our results suggest that the genomic disruption of the DNMTs associated with a MedIP-promoter microarray approach is a useful strategy for “catching” new genes undergoing DNA methylation–associated silencing in human cancer. We have shown that the CpG island hypermethylation of these newly identified genes is not a unique feature of HCT-116 cells, but is also common among colorectal tumorigenesis, which is also found in other colon cancer cell lines, primary colon tumors, and colon adenomas. The list of epigenetically silenced genes covers most of the disrupted pathways of cancer cells (14), such as Ras-mediated signal transduction and development, exemplified by RASGRF2 and HOXD1, respectively. These two cases are not isolated epigenetic events in their categories, but are added to other Ras-related genes such as the Ras effectors RASSF1A and NORE1A (1–3, 7), and homeobox genes such as HOXA9, LMX-1, HOXA5, and DUX-4 (1–3, 7). SCNN1B is another interesting case because it codes for the β subunit of an epithelial sodium channel and epigenetically silencing of other ion channels such as CACNA1G and CALCA11 (1–3, 7) seems to be a common finding in human tumors. These data, and the newly identified epigenetic silencing of SCNN1B in our experiments, are evidence in favor of the proposed role of sodium, calcium, potassium, and chloride channels in the regulation of cell proliferation, migration, and invasion (18). Finally, the identification of CpG island hypermethylation of the BHLB9 gene pinpoints another family of proteins critical to tumorigenesis, the basic helix-loop (bHLH) factors (19). The bHLH family of transcription factors functions in the coordinated regulation of gene expression, cell lineage commitment, and cell differentiation in most tissues (19). In the case of BHLB9, a pivotal role in apoptotic cell death has been proposed (20). Interestingly, BHLB9 hypermethylation is not an isolated event in its category; other bHLH proteins, such as TWIST2 (15), also undergo methylation–associated silencing in human neoplasias. This further underlines the role of this family of transcription factors in cellular transformation.

Thus, overall, we have shown that the use of powerful epigenomic technologies, such as MedIP in conjunction with comprehensive promoter microarrays, in cancer cells whose DNMT genes have been disrupted, could identify new hypermethylated genes in human colorectal tumorigenesis. These aberrantly epigenetically silenced genes are members of the various cellular pathways that contribute to the tumorigenic phenotype and illustrate the disrupted DNA methylation landscape present in cancer cells.

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References

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