Identification of Genes Uniquely Involved in Frequent Microsatellite Instability Colon Carcinogenesis by Expression Profiling Combined with Epigenetic Scanning

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ABSTRACT

Gene silencing through CpG island hypermethylation has been associated with genesis or progression of frequent microsatellite instability (MSI-H) cancers. To identify novel methylation sites unique to MSI-H colon cancers in an unbiased fashion, we conducted a global expression profiling-based methylation target search. We identified 81 genes selectively down-regulated in MSI-H cancers using cDNA microarray analysis of 41 primary colon cancers. Forty six of these 81 genes contained CpG islands overlapping their 5′ untranslated regions. Initial screening of six genes in 57 primary colon cancers detected the following gene with MSI-H cancer-specific hypermethylation: RAB32, a ras family member and A-kinase-anchoring protein, was methylated in 14 of 25 (56%) MSI-H cancers but in none of 32 non-MSI-H cancers or 23 normal colonic specimens. RAB32 hypermethylation correlated with RAB32 mRNA down-regulation and with hMLH1 hypermethylation. In addition, the protein-tyrosine phosphatase receptor type O gene, PTPRO, was frequently methylated in right-sided tumors. This methylation screening strategy should identify additional genes inactivated by epigenetic silencing in colorectal and other cancers.

INTRODUCTION

MSI-H colon cancer, a tumor subset defined by defective DNA mismatch repair, is distinguished by clinical characteristics including poor differentiation, severe inflammatory cell infiltration, proximal anatomical location, and a better prognosis (1, 2). The molecular carcinogenetic pathway underlying MSI-H cancers is distinct from the chromosomal instability pathway underlying non-MSI-H cancers (1), resulting in a unique molecular profile (3). One major factor defining the MSI carcinogenetic pathway is a unique set of tumor suppressor genes, including TGFBR2, BAX, and others, that are inactivated through frameshift mutation at coding region mononucleotide repeats (4, 5). Another distinguishing characteristic of these tumors is the CpG island hypermethylation-mediated silencing of hMLH1, one of the essential components of the DNA mismatch repair system (6). CpG island hypermethylation occurring at promoter regions is frequently associated with transcriptional repression and loss of gene function (7). The significance of altered global CpG island methylation patterns in colon carcinogenesis is under investigation (8, 9). It is possible that hypermethylation-mediated silencing of a subset of tumor-related genes contributes to the unique molecular phenotype of MSI-H cancers. To identify novel genes inactivated through promoter region CpG island hypermethylation in MSI-H colon cancers, we conducted a comprehensive search of promoter region CpG island hypermethylation using a differential gene expression-based approach.

MATERIALS AND METHODS

Tissues and Cell Lines. Eighty-five primary colon cancers (27 MSI-H and 58 non-MSI-H), 14 colon cancer cell lines (5 MSI-H and 9 non-MSI-H), 26 normal colon mucosas, and 4 normal peripheral WBCs from our tissue repository were used for this study. Extractions of genomic DNAs and total RNAs from frozen samples were performed according to standard protocols. Tumor microsatellite instability (MSI) status was determined based on 11 microsatellite markers; i.e., specimens with MSI at ≥30% of informative loci were labeled as MSI-H, whereas specimens showing MSI at <30% of informative loci were labeled as non-MSI-H. The primer sequences for these 11 loci are listed in Supplemental Table 1. The assignment of cases to each experiment was based on the quantity and quality of nucleic acids for each specimen. Three MSI-H and three non-MSI-H primary cancers used for the microarray experiments were also included in the real-time quantitative methylation-specific PCR (rtQ MSP) experiments. Similarly, four MSI-H and 11 non-MSI-H primary cancers in addition to five normal colon mucosas in the rtQ MSP experiments were also included in the real-time quantitative reverse transcription-PCR (rtRT PCR) experiments. The clinicopathological characteristics of the cases are summarized in Table 1.

cDNA Microarray Analyses and Significance Analysis of Microarray (SAM) Data. cDNA microarray analysis and preprocessing of raw data were performed as described previously (3). In brief, 30 μg of total RNA were amplified using a T7-based protocol, and 6 μg of the resulting amplified RNA were labeled with Cy3- or Cy5-labeled dCTP. An amplified RNA pool of human cancer cell lines was used as the reference probe (3). In-house microarray slides containing 8064 sequence-verified human cDNA were used (3). We included in our final analysis only 6242 clones with expression information for at least 96% of the tumors. Global intensity-based normalization was performed by a robust scatter-smoothing method (3). We performed SAM on the normalized microarray data to select genes differentially expressed between MSI-H and microsatellite-stable cancers at a false discovery rate of <0.1.

rtQ MSP. rtQ MSP was performed using the TaqMan system as described previously (10). In brief, bisulfite treatment of genomic DNAs was performed according to the protocol of J. P. Issa.4 A TaqMan primer-probe set to detect the fully methylated allele for each gene was designed within a CpG island overlapping the 5′ untranslated region of the gene. To normalize data, duplex PCR with an α actin (ACTB) primer and probe sequences containing no CpGs was performed. The detailed PCR protocol is described in Supplemental Method 1. CpGenome Universal Methylated DNA (Intergen) was used as a control DNA to generate a standard curve. The ratios for methylated alleles representing the percentage of densely methylated DNA in the sample at the target sequence were calculated as follows:

\[
\text{Ratio for methylated allele} = \frac{(\text{TarS/TarC})}{(\text{ActS/ActC})}
\]

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancers.cancerres.org).

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where TarS and TarC represent levels of target gene methylation in the sample and the control DNA, respectively, and ActS and ActC correspond to the amplified ACTB level in the sample and the control DNA, respectively. Specimens with a methylated allele ratio ≥0.2 were classified as positive for methylation. Primer and probe sequences are listed in Supplemental Table 1.

rtQRTPCR. rtQRTPCR was performed using the TaqMan system, as described previously (10). The template cDNA for each reaction was synthesized from 500 ng of total RNA using SuperScript III kit (Invitrogen) and random hexamer. TaqMan rRNA Control Reagents (Applied Biosystems) were used for normalization of the data. The detailed PCR protocol is described in Supplemental Method 1. cDNA from a colon cancer cell line was used as the control DNA, respectively, and 

Expression index was calculated according to the following formula for the relative expression of target mRNA:

Expression index = (TarS/TarC)\((rRNAS/rRNAC)\)

where TarS and TarC represent levels of mRNA expression for the target gene in the sample and control cDNA, respectively, whereas rRNAS and rRNAC correspond to the amplified rRNA levels in the sample and control cDNA, respectively. The sequences of primers and probes are listed in Supplemental Table 1.

RESULTS

To identify novel targets of hypermethylation-mediated gene silencing in MSI-H colon cancers, we used an approach based on comprehensive scanning of differential gene expression using cDNA microarrays. Initially, we performed a microarray analysis of 12 MSI-H and 29 non-MSI-H primary colon cancers to identify genes significantly down-regulated in MSI-H cancers. Significant down-regulation in MSI-H relative to non-MSI-H cancers was detected by SAM in 81 of 8064 human cDNA clones on our microarrays (Supplemental Table 2). Next, an on-line public database search revealed that 46 of these 81 genes had CpG islands overlapping their 5′-untranslated region or the first exon. Thus, 46 genes were potential targets of hypermethylation-mediated gene silencing unique to MSI-H colon cancers.

We analyzed the methylation status of CpG islands overlapping the 5′untranslated regions of six genes selected from these 46 genes based on putative functional links to human cancers: EFN1B1, ITPR2, HDAC11, PTTPRO, RAB32, and SBDBCA9. A rtQMP assay was performed on 13 primary colon cancers (five MSI-H and eight non-MSI-H), and methylation in tumors was detected in two of the six genes. These two genes, PTTPRO and RAB32, were analyzed further in a larger number of samples consisting of 57 primary cancers, 14 colon cancer cell lines, 23 normal mucosae, and four normal peripheral WBCs. Hypermethylation of RAB32, a Ras family member that encodes a mitochonndrial A-kinase-anchoring protein, was detected in an MSI-H cancer-specific manner; i.e., RAB32 was methylated in 14 (56%) of 25 MSI-H primary cancers but in none of 32 non-MSI-H primary cancers (Fig. 1A; P < 0.00001 by Fisher’s exact test and Student’s t test). Similarly, RAB32 methylation was found in four of five (80%) MSI-H cell lines but in only one (11%) of nine non-MSI-H cell lines. No RAB32 methylation was detected in either 23 normal mucosae or four normal peripheral WBCs. PTTPRO, a membranous protein tyrosine phosphatase gene, was frequently methylated in a tumor-specific manner. PTTPRO methylation was detected in 28 of 55 (55%) primary cancers but in none of 18 normal mucosae (Fig. 1B; P = 0.00003 by Fisher’s exact test and P = 0.0022 by Student’s t test). PTTPRO hypermethylation was more frequent in MSI-H primary cancers (16 of 25, 64%) than in non-MSI-H primary cancers (12 of 30, 40%), but the difference between these two tumor subgroups was not significant.

We further investigated the association between hypermethylation and silenced mRNA expression for RAB32 and PTTPRO. rtQRTTPCR analyses were performed on 14 primary cancers (3 MSI-H and 11 non-MSI-H), 14 cell lines (five MSI-H and nine non-MSI-H), and eight normal mucosae. All eight normal mucosae expressed RAB32 mRNA, MSI-H cancers tended to show lower RAB32 expression than did non-MSI-H cancers and normal colon mucosae, and the results from microarray and rtQRTPCR matched closely among five primary cancers (two MSI-H and three non-MSI-H) analyzed by both methods (data not shown). All five cell lines with RAB32 methylation lacked mRNA expression, and both MSI-H primary cancers with RAB32 methylation showed the lowest mRNA levels among tissue specimens analyzed (data not shown). Fig. 2 illustrates the inverse association between methylation and mRNA expression of the RAB32 gene; i.e., RAB32 mRNA expression was more significantly down-regulated in...
PTPRO mRNA expression was very low or absent in normal colonic tissues. Our microarray results matched closely in six primary cancers (three MSI-H and three MSI-L/N). Ratio for methylated allele was the ratio of the relative quantities of fully methylated genomic DNA for the target gene to the ACTB genomic DNA. The primer and TaqMan probe set for ACTB does not contain any CpGs and amplifies the sample regardless of the methylation status. A chemically modified fully methylated human genomic DNA was used as the quantification standard. All $P$-values were calculated by Student’s $t$-test. A, RAB32 was methylated only in MSI-H tumors, with one exception of a non-MSI-H cancer cell line. B, only cancerous specimens had PTPRO methylation. Tumor MSI status did not show any significant association with the PTPRO methylation level. The dot marked with an asterisk represents a non-MSI-H colon cancer cell line colo205, which had an extremely high methylated allele ratio (4.56).

Specimens with RAB32 hypermethylation than in specimens without RAB32 hypermethylation ($P = 0.0017$; Student’s $t$-test). In accordance with our microarray results, PTPRO mRNA expression levels also tended to be lower in MSI-H primary cancers than in non-MSI-H primary cancers (data not shown), and microarray and qRT-PCR results matched closely in six primary cancers (three MSI-H and three non-MSI-H) analyzed by both methods (data not shown). However, PTPRO mRNA expression was very low or absent in normal colonic specimens and showed no significant association with PTPRO methylation ($P = 0.7354$; Student’s $t$-test; data not shown).

RAB32 hypermethylation was then analyzed for its association with clinicopathological and molecular characteristics of tumors. One of our major interests was to analyze RAB32 hypermethylation in association with hMLH1 hypermethylation, the cause of MSI in the majority of sporadic MSI-H cancers. As shown in Table 2, RAB32 hypermethylation occurred only in specimens with hMLH1 hypermethylation. Furthermore, RAB32 hypermethylation occurred only in tumors with hMLH1 hypermethylation, and levels of hypermethylation of RAB32 and hMLH1 showed a significant linear correlation in 25 MSI-H primary colon cancers (correlation coefficient, 0.907; $P < 0.0001$). RAB32 hypermethylation in MSI-H primary cancers was also significantly associated with right-sided tumor location ($P = 0.0038$, Fisher’s exact test). On the other hand, RAB32 hypermethylation was not associated with histological differentiation, tu

Table 2: RAB32 methylation status of 25 primary MSI-H colon cancers in association with their case or tumor characteristics

<table>
<thead>
<tr>
<th>Category</th>
<th>(+)</th>
<th>(-)</th>
<th>Total</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMLH1 methylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>14</td>
<td>1</td>
<td>15</td>
<td>&lt;0.00001 (Fisher’s exact test)</td>
</tr>
<tr>
<td>(-)</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>correlation coefficient</td>
<td>0.907</td>
<td></td>
<td></td>
<td>($P &lt; 0.0001$)</td>
</tr>
<tr>
<td>Site*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0.0038 (Fisher’s exact test)</td>
</tr>
<tr>
<td>R</td>
<td>14</td>
<td>4</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Histological differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD + MD</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>0.0635 (Fisher’s exact test)</td>
</tr>
<tr>
<td>MPD + PD</td>
<td>9</td>
<td>3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Dukes stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A + B</td>
<td>11</td>
<td>5</td>
<td>16</td>
<td>0.0823 (Fisher’s exact test)</td>
</tr>
<tr>
<td>C + D</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td></td>
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<tr>
<td>Age</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤70</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>0.1906 (Fisher’s exact test)</td>
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<tr>
<td>&gt;70</td>
<td>8</td>
<td>4</td>
<td>12</td>
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</tr>
</tbody>
</table>

*Tumors at transverse colon without detailed description on their position were labeled as NA.

**L, left-sided; R, right-sided; NA, not available; WD, well differentiated; MD, moderately differentiated; MPD, moderately or poorly differentiated; PD, poorly differentiated.
Table 3  PTTPRO methylation status of 55 primary colon cancers in association with their case or tumor characteristics

<table>
<thead>
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<th>(−)</th>
<th>Total</th>
<th>P</th>
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<td>MSI status&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td>H</td>
<td>16</td>
<td>9</td>
<td>25</td>
<td>0.0462 (Fisher’s exact test)</td>
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<tr>
<td>non-H</td>
<td>12</td>
<td>18</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>hMLH1 methylation</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>15</td>
<td>3</td>
<td>18</td>
<td>0.0008 (Fisher’s exact test)</td>
</tr>
<tr>
<td>(−)</td>
<td>13</td>
<td>24</td>
<td>37</td>
<td>0.634 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>Site&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>R</td>
<td>22</td>
<td>7</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>18</td>
<td>21</td>
<td>0.00016 (Fisher’s exact test)</td>
</tr>
<tr>
<td>Histological differentiation&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD + MD</td>
<td>15</td>
<td>20</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>MPD + PD</td>
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<td>5</td>
<td>17</td>
<td>0.0421 (Fisher’s exact test)</td>
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</tr>
<tr>
<td>≤70</td>
<td>12</td>
<td>19</td>
<td>31</td>
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<tr>
<td>&gt;70</td>
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<td>15</td>
<td>33</td>
<td>0.152 (Fisher’s exact test)</td>
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<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> MSI-H, non-H, non-MSI-H

<sup>b</sup> R, right-sided; L, left-sided; NA, not available; WD, well differentiated; MD, moderately differentiated; MPD, moderately to poorly differentiated; PD, poorly differentiated.

DISCUSSION

Comprehensive identification of gene silencing through promoter region hypermethylation has proven fruitful not only for a deeper understanding of human carcinogenesis, but also in the development of biomarkers relevant to the clinical care of patients, because hypermethylation of some genes has been reported as a potential biomarker for early detection or prognostication (11, 12). Several methods of global screening for differentially methylated CpG islands have been described previously (13, 14). Although valuable, these methods were not specific to promoter region hypermethylation or hypermethylation accompanied by gene silencing, which should be necessary in order for a biological effect on cancer progression to be exerted. Therefore, a comprehensive survey based on differential mRNA expression offers the advantage of limiting identification of promoter region hypermethylation events to those correlated with gene silencing only (15).

In the current study, we combined methylation target scanning with differentially expressed gene screening by cDNA microarrays to identify novel targets of hypermethylation-mediated gene silencing in MSI-H colon cancers. By using this strategy, we identified 46 genes as candidate targets, and a detailed analysis of 6 of these 46 genes successfully identified a novel target of MSI-H tumor-specific hypermethylation that correlated with gene silencing. This novel target, RAB32, is a mitochondrial small molecular weight G-protein that belongs to the Ras superfamily and participates in synchronization of mitochondrial fission (16). RAB32 is distinct from other RAB family proteins by its characteristics as an A-kinase-anchoring protein, i.e., the ability to anchor the cyclic AMP-dependent kinase (PKA) by binding to the regulatory subunit of PKA (16). Therefore, RAB32 inactivation may alter the phenotype of the cell through disruption or dysregulation of one or more cyclic AMP-PKA-mediated cellular functions in or around mitochondria, including apoptosis and energy production (17, 18).

RAB32 hypermethylation was associated only with MSI-H, or more specifically hMLH1 promoter region hypermethylation, among all tumor characteristics analyzed in this study. An observed association with right-sided tumor location probably arose from the fact that MSI-H cancers are predominantly right-sided. Aging did not appear to increase the hypermethylation rate for RAB32, although aging is known to affect methylation rates of several genes (19). The observed close correlation between hMLH1 and RAB32 methylation suggests that cancers caused by hMLH1 methylation form a subgroup with a slightly different molecular phenotype in MSI-H cancers. A previous report on differences in features between hereditary nonpolyposis colon carcinoma and sporadic MSI-H cancers supports this hypothesis (20). Another molecular abnormality associated with hMLH1 hypermethylation is activating mutation of BRAF, which seems to functionally compensate for the lack of KRAS mutation in MSI-H cancers (21, 22). Interestingly, PKA also participates in the RAS/RAF/mitogen-activated protein kinase-extracellular signal-regulated kinase extracellular signal-regulated kinase cascade as a modulator (23), suggesting that PKA is a potential link between BRAF and RAB32. Additional functional studies are required to scrutinize the role of RAB32 in both these signaling pathways and the genesis of human cancers.

The protein tyrosine phosphatase receptor type O gene, PTTPRO, was another gene displaying frequent tumor-specific hypermethylation but not in an MSI-H-specific manner. Recently, DNA hypermethylation-mediated silencing of PTTPRO was reported in rat hepatocellular cancers induced by a folic acid-deficient diet (24). In contrast to rat liver, gene silencing by PTTPRO hypermethylation was not observed in human colon. However, PTTPRO hypermethylation in colon cancers showed a remarkable association with right-sided tumor location independent of tumor MSI status. Interestingly, diet has been suggested as one of the modifiers of DNA methylation patterns and is known to have a prominent influence on the intraluminal contents of the right hemicolon (25). Thus, PTTPRO hypermethylation could conceivably constitute a marker of diet-related alterations in colonic methylation pattern.

In summary, we have presented an application of differential expression scanning with cDNA microarrays to the comprehensive identification of promoter region hypermethylation-mediated gene silencing in human cancer. In proof of principle, a novel methylation target specific to MSI-H colon cancers was identified using this approach. This novel target, RAB32, participates in the cyclic AMP-PKA signaling pathway and is potentially relevant to a subset of MSI-H cancer progression. These findings suggest that this combined strategy offers the potential to discover additional methylation events unique to other specific cancer subgroups, and in doing so, to provide insights into the unique biology underlying these tumor subgroups.

REFERENCES


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