Mismatch Repair Deficiency and CpG Island Hypermethylation in Sporadic Colon Adenocarcinomas 1

Zhenggang Xiong, Anna H. Wu, Christina M. Bender, Jen-Lan Tsao, Corey Blake, Darryl Shibata, Peter A. Jones, Mimi C. Yu, Ronald K. Ross, and Peter W. Laird 2


Abstract
Many studies have documented CpG island hypermethylation in human colon adenocarcinomas. Several of these reports have additionally found such CpG island hypermethylation to be more extensive in tumors with a mismatch-repair-deficiency, as revealed by microsatellite instability (MSI+). Because the source of samples used in these prior studies may not have been representative of the general population, we have reinvestigated this issue using samples from a population-based study. A total of 15 MSI+ tumors were identified, and they were compared with 47 MSI− tumors that were similar in distribution by age, sex, and race. Microdissected tumor and normal adjacent mucosal DNA samples from each patient were subjected to a quantitative DNA methylation analysis at 13 separate CpG dinucleotides located in five CpG islands in four different genes [APC, ESR1 (ER), CDKN2A (p16; promoter and exon 2), and MLH1]. Four of five CpG islands showed a statistically significantly increased level of methylation in tumor tissue compared with adjacent normal mucosa. In contrast to previous studies, we did not find any statistically significant correlations between MSI status and methylation levels of any of the CpG islands other than MLH1. Furthermore, we observed a positive correlation between MLH1 methylation and CDKN2A methylation (P = 0.03), whereas no association was noted between MSI positivity and CDKN2A methylation (P = 0.95). The latter results suggest a possible defect in the protection against CpG island hypermethylation shared between CDKN2A and MLH1 and do not support the notion of a functional association between CDKN2A methylation and the phenotype of mismatch repair deficiency.

Introduction
Transcriptional silencing of genes by DNA hypermethylation of associated CpG islands has been documented in a diversity of human neoplasms, including colorectal adenocarcinomas (1, 2). Recently, it has been proposed that a subset of colorectal tumors show unusually widespread CpG island hypermethylation (3) and that this methylator phenotype (CIMP+) is statistically significantly associated with MSI+. 3 In these tumors (3, 4), DNA methylation patterns in human colorectal tumors and normal mucosa are known to be influenced by pathological and demographic criteria, such as tumor colonic subsite, age, and gender (5, 6). Therefore, we have investigated the link between CpG island hypermethylation and MSI+ in an ongoing population-based cross-sectional study of colon cancer in Los Angeles County.

Materials and Methods
Patient Selection. Patients in this study were participants in an ongoing population-based incidence study of colon cancer in Los Angeles County, which was designed to determine the population prevalence, including age-, gender-, and race-specific variation, of phenotypically MSI+ colon cancers. In brief, the parent study randomly sampled approximately 5% of subjects 70–79 years of age and 10% of subjects 60–69 years of age and selected all of the subjects <60 years of age who were diagnosed with an incident, histologically confirmed adenocarcinoma of the colon or after August 1, 1995 until 500 subjects had been recruited. Race and gender distributions of colon cancer patients recruited into the parent cross-sectional study were representative of all of the eligible colon cancer patients identified during the study period by the Cancer Surveillance Program, the population-based Surveillance, Epidemiology, and End Results (SEER) cancer registry for Los Angeles County. An in-person interview was conducted with studied subjects in English and asked extensive questions on known and suspected lifestyle risk factors and family history of cancer. In addition, subjects were asked to donate a blood specimen and to sign a consent form that authorized the release of medical record information and tumor specimens. Ninety-four% of the subjects interviewed consented to the release of tumor materials from which MSI status was determined. Comparable age, gender, and race distributions were found between those who consented and those who did not consent to the release

1 The abbreviations used are: MSI, microsatellite instability; CIMP, CpG island methylator phenotype; COBRA, combined bisulfite restriction analysis; MS-SNuPE, methylation-sensitive single nucleotide primer extension; MSP, methylation-specific PCR.

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of tumor materials. From the first 160 colon cancer patients participating in the parent study who were tested for MSI status, we included in this methylation study the 15 subjects who were classified as MSI− and an additional 47 patients who were classified as MSI+. The MSI− cases were selected based on frequency matching to the MSI+ cases on age (10-year age group), gender, and race (Caucasian versus non-Caucasian). All but one of the 15 MSI+ tumors used in this study were considered to be sporadic. Only one patient fulfilled the Amsterdam criteria for hereditary nonpolyposis colorectal cancer (7).

**Microdissection and DNA Extraction.** Paraffin sections from diagnosis/resection were obtained for each of the 62 cases. Adenocarcinoma tissue was identified and microdissected, along with adjacent, histologically normal tissue. Determination of MSI status and DNA methylation levels of CpG islands were conducted using the DNA extracted from paraffin-embedded tumor blocks. The investigators conducting the methylation analysis were blinded to the MSI status and demographic and tumor characteristics of the subjects. Paraffin sections were lightly stained with H&E or acetate slides. Areas of tumor and normal mucosa were identified by a pathologist (D. S.) and dissected separately by cutting the acetate slide and attached tissue with scissors. DNA was extracted by overnight incubation of the dissected tissue in 100 μl of lysin buffer [100 mM Tris-HCl, 2 mM EDTA (pH 8.0), and 400 μg/ml proteinase K] at 42°C as described (8). The samples were denatured for 5–7 min at 100°C before bisulfite conversion.

**MSI Analysis.** MSI status was determined by PCR of the BAT25 and BAT26 loci as described previously (9) and by PCR of dinucleotide CA-repeat microsatellites located in noncoding genomic regions (HSMfd27, HSMfd41, HSMfd47, and HSMfd57; Ref. 10). PCR was performed in 96-well plates in a genomic regions (HSMfd27, HSMfd41, HSMfd47, and HSMfd57; Ref. 10). PCR was performed in 96-well plates in a volume of 10 μl. PCR products were labeled by incorporating [32P]dCTP (added at the start). Electrophoresis of the PCR products was performed on 6% polyacrylamide gels. The size of each microsatellite was determined by comparing normal tissue alleles. Samples were considered to be MSI+ if both BAT25 and BAT26 and at least two of the four CA-repeats showed evidence of MSI.

**DNA Methylation Assays.** DNA methylation levels at five CpG islands (APC, CDKN2A promoter and exon 2, ESR1, and MLH1) were determined quantitatively using either COBRA (11) or Ms-SNuPE (12). These two techniques both perform methylation analysis. The choice of assay in each case was dictated by the ease of design.

**Statistical Analysis.** Each subject was assigned two scores for the percentage methylation at each of the five CpG islands, one for each of the adenocarcinoma tissue and one for the adjacent normal mucosal tissue, respectively. For each of the genes studied, because more than one individual CpG dinucleotide located in the five CpG islands was tested for the percentage DNA methylation, an average methylation level of CpG island was computed (three CpGs for APC, two for ESR1, three for CDKN2A promoter, three for CDKN2A exon 2, and two for MLH1). In addition, we constructed a summary methylation score for the CDKN2A gene by adding the respective rankings of methylation scores at the promoter and exon 2 loci. The distribution of the percentage methylation was markedly skewed for each gene of interest. Therefore, nonparametric statistical methods and parametric methods applied to logarithmically transformed variables were used to analyze the data, and geometric (as opposed to arithmetic) means and their associated 95% confidence intervals are presented in this report. Specifically, the Wilcoxon rank-sum test was used to compare the percentage methylation for selected genes in colorectal tumors by anatomical subsite, race, and gender; (b) to compare the percentage methylation for selected genes in MSI− colorectal tumors; and (c) to compare the percentage methylation for selected genes, including the percentage methylation score for the CDKN2A gene (see above), among tumors displaying high (above the median) versus low (below the median) MLH1 methylation (13). The Spearman rank order correlation coefficient was used to examine the relationship between the percentage methylation and age (13). The Wilcoxon signed rank test was used to compare (a) ratio of the percentage methylation and (b) arithmetic difference in the percentage methylation for selected genes in tumor versus normal colorectal tissues in study subjects (13). Because age was shown to correlate significantly with the percentage methylation for the ESR1 gene in tumor tissues, comparison for this gene between MSI+ and MSI− tumors and between MLH1 high versus MLH1 low methylation was performed using the analysis of covariance method (with age
The regression covariate applied to logarithmically transformed ESR1 percentage methylation (13). All of Ps quoted are two-sided; they are considered to be statistically significant when the values are below 0.05.

Results and Discussion

CpG Island Methylation Levels in Tumor Tissue by Anatomical Subsite and Demographic Characteristics. We first determined whether there were any statistically significant differences in tumor DNA methylation levels by colonic subsite, age, race, or gender (Table 1). Table 1 shows that the percent methylation for the APC, CDKN2A, and MLH1 genes did not differ significantly by anatomical subsite, age, race, or gender. For the ESR1 gene, there was a statistically significant positive correlation between age and the percentage methylation for the five CpG islands in tumor tissue and presence of methylation in tumor tissue. There were no significant differences in the percentage methylation for the APC, CDKN2A, and ESR1 genes in tumor tissue as a function of age, race, or gender. The percentage methylation levels of these four CpG islands in tumor tissues versus normal tissues were independent of MSI status (Table 2). Among MSI− tumors, there was no difference in DNA methylation levels between tumor and normal mucosa at the MLH1 promoter loci (P = 0.63). However, among MSI+ tumors only, a statistically significant difference in the percentage methylation between tumor and normal mucosa was observed (P = 0.04). This latter observation is consistent with the report of Kuismanen et al. (17), who found varying results between MSI+ and MSI− tumors when MLH1 methylation patterns in normal and tumorous colonic tissues were compared. In the Kuismanen et al. study (17), over 60% of the MSI+ colon cancers showed absence of MLH1 methylation in normal tissue and presence of methylation in tumor tissue, whereas some 53% of the MSI− cases displayed methylation in normal mucosa and absence of methylation in tumor DNA.

CpG Island Methylation Levels in Normal versus Tumor Tissue. Table 2 shows the geometric mean (95% confidence intervals) of the percentage methylation for the five CpG islands in tumor versus normal colorectal tissues. It also gives the two-sided Ps in the paired comparisons of the percentage methylation in tumor versus normal tissues. Methylation levels for the APC, CDKN2A (promoter and exon 2), and ESR1 genes in tumor were statistically significantly higher than their counterpart in normal colorectal tissue. These results are consistent with reports published previously (3, 4, 6, 14–16). The higher methylation levels of these four CpG islands in tumor tissues versus normal tissues were independent of MSI status (Table 2).

Table 1

<table>
<thead>
<tr>
<th>Colon subsite</th>
<th>APC (promoter)</th>
<th>CDKN2A (exon 2)</th>
<th>ESR1</th>
<th>MLH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right (n = 27)</td>
<td>7.3 (4.3, 11.9)</td>
<td>3.9 (2.5, 5.9)</td>
<td>31.3 (27.6, 35.4)</td>
<td>24.8 (17.5, 35.0)</td>
</tr>
<tr>
<td>Left (n = 35)</td>
<td>7.4 (4.8, 11.2)</td>
<td>3.8 (2.6, 5.4)</td>
<td>31.4 (27.9, 35.3)</td>
<td>30.9 (24.0, 39.7)</td>
</tr>
<tr>
<td>Age &lt;60 (n = 39)</td>
<td>P = 0.91</td>
<td>0.99</td>
<td>0.72</td>
<td>0.20</td>
</tr>
<tr>
<td>Age ≥60 (n = 23)</td>
<td>5.8 (3.3, 9.6)</td>
<td>3.3 (1.7, 5.7)</td>
<td>29.8 (25.6, 34.6)</td>
<td>34.7 (27.5, 43.9)</td>
</tr>
<tr>
<td>Gender</td>
<td>0.76</td>
<td>0.24</td>
<td>0.57</td>
<td>0.37</td>
</tr>
<tr>
<td>Caucasian (n = 36)</td>
<td>7.2 (4.5, 11.1)</td>
<td>4.1 (2.8, 5.8)</td>
<td>30.1 (27.0, 33.7)</td>
<td>31.2 (24.1, 40.2)</td>
</tr>
<tr>
<td>Non-Caucasian (n = 26)</td>
<td>7.6 (4.7, 12.0)</td>
<td>3.5 (2.2, 5.2)</td>
<td>33.1 (29.1, 37.7)</td>
<td>24.3 (17.2, 34.2)</td>
</tr>
<tr>
<td>Male (n = 20)</td>
<td>8.5 (5.0, 14.1)</td>
<td>4.3 (2.4, 7.2)</td>
<td>33.4 (28.3, 39.5)</td>
<td>25.5 (17.9, 36.4)</td>
</tr>
<tr>
<td>Female (n = 42)</td>
<td>6.8 (4.5, 10.3)</td>
<td>3.6 (2.6, 4.9)</td>
<td>30.4 (27.6, 33.5)</td>
<td>29.4 (22.7, 37.9)</td>
</tr>
</tbody>
</table>

P* for the associations of methylation percentages with age were based on the test of Spearman rank order correlation coefficient = 0.0.

Table 2

<table>
<thead>
<tr>
<th>CpG island</th>
<th>Normal tissue (n = 62)</th>
<th>Tumorous tissue (n = 62)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>3.3 (2.4, 4.4)</td>
<td>7.4 (5.3, 10.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CDKN2A (promoter)</td>
<td>2.3 (1.7, 3.0)</td>
<td>3.8 (2.9, 5.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CDKN2A (exon 2)</td>
<td>26.4 (24.0, 29.0)</td>
<td>31.4 (28.8, 34.1)</td>
<td>0.0002</td>
</tr>
<tr>
<td>ESR1</td>
<td>7.0 (4.8, 10.2)</td>
<td>28.1 (22.8, 34.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MLH1 (All)</td>
<td>9.7 (7.5, 12.3)</td>
<td>9.1 (7.0, 11.7)</td>
<td>0.09</td>
</tr>
<tr>
<td>MLH1 (MSI−)</td>
<td>9.5 (7.1, 12.6)</td>
<td>7.5 (5.8, 9.6)</td>
<td>0.63</td>
</tr>
<tr>
<td>MLH1 (MSI+)</td>
<td>10.2 (6.2, 16.4)</td>
<td>16.7 (9.0, 30.2)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

P* for ratio differences in methylation percentages between normal and tumor colon tissues were based on the Wilcoxon signed rank test.
scores for the CDKN2A gene were summed to form a combined methylation score for this gene (see “Materials and Methods”), a statistically significant association was observed with MLH1 methylation (P = 0.03). CDKN2A methylation based on this combined rank, on the other hand, did not differ significantly between MSI+ and MSI− colorectal tumors (P = 0.95). We also investigated whether the association between MLH1 methylation and CDKN2A methylation clustered in the subgroup of patients with MSI+ tumors and high MLH1 methylation levels (i.e., above median). The combined CDKN2A methylation score in tumor tissues of subjects with MSI+ tumors and high MLH1 methylation scores (n = 11) did not differ significantly (P = 0.18) from other subjects (i.e., four MSI+ tumors with low MLH1 methylation and 47 MSI−). Again, these results suggest that MLH1 methylation rather than MSI status was responsible for the observed difference in CDKN2A methylation.

We conclude that of the five CpG islands analyzed in this study, the association of CpG island hypermethylation with MSI is limited to the MLH1 promoter CpG island. Our study included the CDKN2A promoter CpG island, which is one of the CpG islands that has been associated with MSI+ in other studies (3, 6, 15). The discrepancy between our results and those of other studies may be explained, in part, by differences in study design and methylation analysis technology.

The 62 colorectal cancers (15 MSI+ and 47 MSI−) included in this analysis were derived from a well-characterized population-based series of colon cancers in Los Angeles County. The prevalence of MSI+ (i.e., 15 MSI+ of 160 colorectal cancers tested) in our study is consistent with the rate of MSI+ tumors (10–15%) from other population-based studies of sporadic colorectal cancers (18–20). In contrast, considerably higher proportions of MSI+ tumors (30% or higher) have been reported in hospital-based (15) and other nonpopulation-based studies of colorectal cancers (3, 4) that noted global patterns of CpG island methylation in association with MSI status. The Finnish population-based study (19) used a study design that is more comparable with ours and, in fact, yielded similarly null results on methylation and MSI status (17).

The second methodological difference between previous studies and our study relates to the experimental methods used to determine methylation status. Most previous studies relied on nonquantitative approaches, such as MSP (21), whereas we used two quantitative procedures, COBRA (11) and Ms-SNuPE (12) in the present investigation. Our analyses yielded accurate quantitative data for each individual CpG dinucleotide. On the other hand, techniques such as MSP can sensitively detect extensively methylated molecules that may be present at very low levels in a pool of DNA molecules with mixed methylation patterns. If the presence of such extensively methylated molecules were more strongly associated with MSI+ status than is the mean methylation level of any individual CpG dinucleotide in a pool of mixed DNA molecules, then this could lead to divergent results caused by these differing methodologies. In theory, such a discrepancy between results obtained with a single CpG method versus an allele-specific method such as MSP would exist only in DNA samples containing pools of molecules with mixed patterns of methylation. The heterogeneity associated with primary tissue samples is substantially lower in cultured cell lines. We have found that MSI+ colorectal cancer cell lines do not show increased CpG island hypermethylation at endogenous gene loci, including CDKN2A promoter and exon 2, ESR1, and APC, lending further support to our current results (22).

Although we did not confirm the finding by others (3, 6, 15) of a statistically significant association between CDKN2A and MSI, we did observe significant correlations between overall CDKN2A methylation, as well as specific CDKN2A exon 2 methylation and hypermethylation of the promoter of the MLH1 mismatch repair gene. Our observed association of CDKN2A methylation with MLH1 methylation and lack thereof with MSI positivity suggests that the previously observed link between CDKN2A methylation and MSI may have been an indirect consequence of a methylation defect that leads to the hypermethylation of a specific class of CpG islands that includes both CDKN2A exon 2 and the MLH1 promoter. In other words, shared structural characteristics of the CpG islands, rather than the phenotypic consequences of gene silencing, may be responsible for the concordant methylation behavior of these two CpG islands. Hypermethylation of the exon 2 CpG island of CDKN2A is thought to have little effect on CDKN2A gene expression, whereas promoter methylation leads to silencing of the CDKN2A gene (23, 24). In our study, MLH1 methylation was actually more closely associated with CDKN2A exon 2 methylation than with CDKN2A promoter methylation, which supports the case for shared structural or chromatin features being responsible for the association between methylation of these two genes, rather than the phenotypic results of gene silencing. We conclude that the nature of the relationship between MSI status, MLH1 methylation, and hypermethylation of other CpG islands in sporadic human colorectal adenocarcinomas has not been fully resolved and will require further investigation.

Table 3  Geometric means (95% confidence intervals) of percentage methylation for selected genes in colon tumor tissue by MSI+ status and by high vs. low levels of MLH1 methylation

<table>
<thead>
<tr>
<th>CpG island</th>
<th>MSI status</th>
<th>MLH1 methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 15)</td>
<td>(n = 31)</td>
</tr>
</tbody>
</table>
| APC        | 7.1 (3.9 – 12.3) | 7.4 (5.0 – 10.9) | 6.6 (3.8 – 10.9) | 0.88  
| CDKN2A promoter | 3.9 (1.9 – 7.3) | 3.8 (2.8 – 5.1) | 2.9 (1.9 – 4.3) | 0.77d  
| CDKN2A exon 2 | 32.5 (29.1 – 36.3) | 31.0 (27.9 – 34.5) | 29.0 (26.0 – 32.3) | 0.60d  
| ESR1 | 22.6 (14.3 – 35.4) | 30.1 (23.9 – 37.9) | 26.3 (18.7 – 36.9) | 0.34  
| MLH1 | 16.7 (9.0 – 30.2) | 7.5 (5.8 – 9.6) | NA           | 0.02  

*Ps for differences in methylation percentages between MSI+ and MSI− colorectal tumors were based on the Wilcoxon rank-sum test. 

Low MLH1 methylation status is defined as below median methylation levels (below 8%) and high MLH1 methylation is defined as above median MLH1 methylation levels (8% and above). 

The Ps for differences in methylation percentages between low MLH1 methylation and high MLH1 colon cancers were based on the Wilcoxon rank-sum test. 

The Ps for difference in the combined methylation score for CDKN2A (see “Materials and Methods”) between MSI+ versus MSI− tumors was P = 0.95. 

The Ps for difference in the combined methylation score for CDKN2A (see “Materials and Methods”) between low (below median) versus high (above median) MLH1 methylation levels was P = 0.03. 

NA, not applicable.
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References
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