Short Communication

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]

Departments of Surgery [Z. X., P. W. L.], Biochemistry and Molecular Biology [Z. X., C. M. B., P. A. J., P. W. L.], Preventive Medicine [A. H. W., M. C. Y., R. K. R.], Pathology [J-L. T., C. B., D. S.], and Urology [C. M. B., P. A. J.], University of Southern California Keck School of Medicine, Norris Comprehensive Cancer Center, Los Angeles, California 90089-9176

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 on 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

Received 12/1/00; revised 4/13/01; accepted 5/7/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by Grants N01-CN-67010 and R01-CA-75090 from the National Cancer Institute.
[2] To whom requests for reprints should be addressed, at USC/Norris Comprehensive Cancer Center, Room 6418, 1441 Eastlake Avenue, Los Angeles, CA 90089-9176. Phone: (323) 865-0650; Fax: (323) 865-0158; E-mail: plaird@hsc.usc.edu.

[3] 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.

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 on 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 lysis 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 volume of 10 μl. PCR products were labeled by incorporating [^{33}P]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 (Ref. 11; *APC* and *ESR1*) or Ms-SNuPE (Ref. 12; *CDKN2A* and *MLH1*). Salmon sperm DNA (1 μg) was added as a carrier before the bisulfite treatment of the genomic DNA as described previously (11). PCR amplification of the bisulfite converted DNA was performed using the following primers: *APC*: 5′-AGAGAGAAGTAGTTGTGTTAAT-3′, 5′-ACTACACCAATACAACCACAT-3′; *CDKN2A* promoter: 5′-GTAGGTGGGGAGGAGTTTAGTT-3′, 5′-TCTAATAACCAACCAACCCCTCC-3′; *CDKN2A* exon 2: 5′-TTGATTATTTTGTTTTTTTTGGTAGGTT-3′, 5′-CAAATTCTCAAATCATCAATCCTCACC-3′; *ESR1*: 5′-TCCTAAAACTACACTTACTCC-3′, 5′-GGTTATTTGGAAAAAGAGTATAG-3′; and *MLH1*: 5′-GGAGGTTATAAGAGTAGGGTTAA-3′, 5′-CCAACCAATAAAAACAAAAATACC-3′. For COBRA analyses, the PCR products were digested by restriction endonucleases *Taq*I and SfaNI (*APC*) or *Taq*I and BstUI (*ESR1*). The digested PCR products were electrophoresed on denaturing polyacrylamide gels and electroblotted (11). The membranes were hybridized by a 5′-end-labeled oligonucleotide to visualize both digested and undigested DNA fragments. The *APC* probe was 5′-CCCACACCCAACCAAT-3′. The *ESR1* probe used was 5′-AAACCAAAACTC-3′. Ms-SNuPE reactions (25 ml; Ref. 12) were performed as follows: ~10–50 ng of PCR product template, 1 × Taq polymerase PCR buffer, 1 mM of each Ms-SNuPE primer, 1 mCi of either [^{32}P]dCTP or [^{32}P]dTTP, and 1 unit of Taq polymerase mixed 1:1 with Taq/Taq start antibody (Clontech, Palo Alto, CA). NaOH (0.4 N) was added to terminate the reaction and to denature the DNA before membrane transfer. The Ms-SNuPE primers are located immediately 5′ of the CpG to be analyzed. The following primer sequences were used: *CDKN2A* promoter primer sequences: 5′-TTTGAGGGATAGGGT-3′, 5′-TTTTAGGGGTGTTATATT-3′, 5′-TTTTTTTGTTTGGAAAGATAT-3′; *CDKN2A* exon 2: 5′-GTTGGTGGTGTTGTAT-3′, 5′-AGGTTATGATGATGGGTAG-3′, 5′-TATTAGAGGTAGTAATTATGTT-3′; and *MLH1*: 5′-TAAGGGGAGAGGAGGAGTTTGAGAAG-3′, 5′-TTTAGTAGAGGTATATAAGTT-3′. A pair of reactions was set up for each sample using either [^{32}P]dCTP or [^{32}P]dTTP for single nucleotide extension. The extended Ms-SNuPE primers were separated by denaturing polyacrylamide gel. Phosphorimaging quantitation and calculation of the percentage methylation was performed as described for both COBRA (11) and Ms-SNuPE (12). These two techniques both yield quantitatively accurate results for individual CpG dinucleotide 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 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/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 (*a*) 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+ *versus* MSI− colorectal tumors; and (*c*) to compare the percentage methylation for selected genes, including the summary 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

Table 1 Geometric means (95% confidence intervals) of percentage methylation in colon adenocarcinomas for selected genes according to cancer subsite, age, race, and gender

	APC	CDKN2A (promoter)	*CDKN2A* (exon 2)	*ESRI*	MLH1
Colon subsite					
Right (n = 27)	7.3 (4.3, 11.9)	3.9 (2.5, 5.9)	31.3 (27.6, 35.4)	24.8 (17.5, 35.0)	10.8 (6.9, 16.5)
Left (n = 35)	7.4 (4.8, 11.2)	3.8 (2.6, 5.4)	31.4 (27.9, 35.3)	30.9 (24.0, 39.7)	8.0 (5.9, 10.8)
P^a	0.91	0.99	0.72	0.20	0.27
Age					
<60 (n = 39)	8.4 (5.5, 12.6)	4.2 (3.1, 5.5)	32.4 (29.2, 35.8)	24.7 (18.4, 33.1)	9.1 (6.7, 12.2)
60+ (n = 23)	5.8 (3.3, 9.6)	3.3 (1.7, 5.7)	29.8 (25.6, 34.6)	34.7 (27.5, 43.9)	9.1 (5.6, 14.5)
P^b	0.76	0.24	0.28	0.02	0.38
Race					
Caucasian (n = 36)	7.2 (4.5, 11.1)	4.1 (2.8, 5.8)	30.1 (27.0, 33.7)	31.2 (24.1, 40.2)	9.4 (6.8, 12.8)
Non-Caucasian (n = 26)	7.6 (4.7, 12.0)	3.5 (2.2, 5.2)	33.1 (29.1, 37.7)	24.3 (17.2, 34.2)	8.7 (5.6, 13.3)
P^a	0.83	0.64	0.57	0.14	0.67
Gender					
Male (n = 20)	8.5 (5.0, 14.1)	4.3 (2.4, 7.2)	33.4 (28.3, 39.5)	25.5 (17.9, 36.4)	10.0 (6.3, 15.5)
Female (n = 42)	6.8 (4.5, 10.3)	3.6 (2.6, 4.9)	30.4 (27.6, 33.5)	29.4 (22.7, 37.9)	8.7 (6.3, 11.9)
P^a	0.53	0.60	0.56	0.47	0.80

[a] *P*s for differences in methylation percentages by subsite, race, and gender were based on the Wilcoxon rank-sum test.
[b] *P*s for the associations of methylation percentages with age were based on the test of Spearman rank order correlation coefficient = 0.0.

as the regression covariate) applied to logarithmically transformed *ESR1* percentage methylation (13). All of *P*s 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 percentage 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 (P = 0.02). No relationship was found for *ESR1* with tumor subsite, race, or gender.

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 *P*s 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).

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.

Table 2 Geometric means (95% confidence intervals) of percentage methylation for selected genes in colonic normal and tumor tissues

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

[a] *P*s for ratio differences in methylation percentages between normal and tumor colonic tissues were based on the Wilcoxon signed rank test.

CpG Island Hypermethylation by MSI Status and by *MLH1* Methylation in Tumors. Table 3 presents the geometric means (95% confidence intervals) of the percentage methylation for the four studied genes in MSI+ *versus* MSI− tumors. There were no significant differences in the percentage methylation between MSI+ and MSI− tumors for the *APC*, *CDKN2A*, and *ESR1* genes. In contrast, there was a statistically significant difference in the percentage methylation for the *MLH1* gene between MSI+ *versus* MSI− tumors (P = 0.02).

Table 3 also presents the geometric means (95% confidence intervals) of the percentage methylation for the four other CpG islands [*i.e.*, *APC*, *CDKN2A* (promoter and exon 2), and *ESR1* genes] for colon cancers exhibiting low (below median) *versus* high (above median) *MLH1* methylation levels. The percentage methylation of *APC* and *ESR1* genes did not differ between tumors classified as showing low *versus* high *MLH1* methylation levels. On the other hand, the percentage methylation of both *CDKN2A* promoter (P = 0.11) and exon 2 (P = 0.03) were higher among colon tumors exhibiting high *MLH1* methylation levels compared with those showing low *MLH1* methylation levels. When the rankings of these two methylation

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

CpG island	MSI status		P^a	*MLH1* methylation[b]		P^c
	MSI+ ($n = 15$)	MSI− ($n = 47$)		Low (< median) ($n = 31$)	High (> median) ($n = 31$)	
APC	7.1 (3.9, 12.3)	7.4 (5.0, 10.9)	0.88	6.6 (3.8, 10.9)	8.2 (5.5, 12.1)	0.38
CDKN2A promoter	3.9 (1.9, 7.3)	3.8 (2.8, 5.1)	0.77[d]	2.9 (1.9, 4.3)	4.9 (3.3, 7.1)	0.11[e]
CDKN2A exon 2	32.5 (29.1, 36.3)	31.0 (27.9, 34.5)	0.60[d]	29.0 (26.0, 32.3)	34.0 (29.9, 38.5)	0.03[e]
ESR1	22.6 (14.3, 35.4)	30.1 (23.9, 37.9)	0.34	26.3 (18.7, 36.9)	29.9 (23.5, 38.1)	0.42
MLH1	16.7 (9.0, 30.2)	7.5 (5.8, 9.6)	0.02	NA[f]	NA	NA

[a] *P*s for differences in methylation percentages between MSI+ and MSI− colon cancers were based on the Wilcoxon rank-sum test.
[b] 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).
[c] *P*s for differences in methylation percentages between low *MLH1* methylation and high *MLH1* colon cancers were based on the Wilcoxon rank-sum test.
[d] The *P*s for difference in the combined methylation score for *CDKN2A* (see "Materials and Methods") between MSI+ *versus* MSI− tumors was $P = 0.95$.
[e] The *P*s 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 $P = 0.03$.
[f] NA, not applicable.

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− colon 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+ 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 found to be 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 colon 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 colon 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.

Acknowledgments

We thank Kazuko Arakawa for her assistance in data analysis.

References

1. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv. Cancer Res., *72:* 141–196, 1998.

2. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.

3. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. CpG island methylator phenotype in colorectal cancer. Proc. Natl. Acad. Sci. USA, *96:* 8681–8686, 1999.

4. Ahuja, N., Mohan, A. L., Li, Q., Stolker, J. M., Herman, J. G., Hamilton, S. R., Baylin, S. B., and Issa, J. P. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res., *57:* 3370–3374, 1997.

5. Ahuja, N., Li, Q., Mohan, A. L., Baylin, S. B., and Issa, J. P. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res., *58:* 5489–5494, 1998.

6. Wiencke, J. K., Zheng, S., Lafuente, A., Lafuente, M. J., Grudzen, C., Wrensch, M. R., Miike, R., Ballesta, A., and Trias, M. Aberrant methylation of p16INK4a in anatomic and gender-specific subtypes of sporadic colorectal cancer. Cancer Epidemiol. Biomark. Prev., *8:* 501–506, 1999.

7. Vasen, H. F., Mecklin, J. P., Khan, P. M., and Lynch, H. T. The International Collaborative Group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). Dis. Colon Rectum, *34:* 424–425, 1991.

8. Shibata, D. The polymerase chain reaction and the molecular genetic analysis of tissue biopsies. *In:* C. S. Herrington and J. O. D. McGee (eds.), Diagnostic Molecular Pathology: A Practical Approach, Vol. II, pp. 85–111. Oxford: IRL Press, 1992.

9. Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. Microsatellite instability and mutations of the transforming growth factor-β type II receptor gene in colorectal cancer. Cancer Res., *55:* 5548–5550, 1995.

10. Shibata, D., Navidi, W., Salovaara, R., Li, Z. H., and Aaltonen, L. A. Somatic microsatellite mutations as molecular tumor clocks. Nat. Med., *2:* 676–681, 1996.

11. Xiong, Z., and Laird, P. W. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res., *25:* 2532–2534, 1997.

12. Gonzalgo, M. L., and Jones, P. A. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res., *25:* 2529–2531, 1997.

13. Snedecor, G. W., and Cochran, W. G. Statistical Methods. Ames: Iowa State University Press, 1989.

14. Hiltunen, M. O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V. M., and Janne, J. Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. Int. J. Cancer, *70:* 644–648, 1997.

15. Liang, J. T., Chang, K. J., Chen, J. C., Lee, C. C., Cheng, Y. M., Hsu, H. C., Wu, M. S., Wang, S. M., Lin, J. T., and Cheng, A. L. Hypermethylation of the p16 gene in sporadic T3N0M0 stage colorectal cancers: association with DNA replication error and shorter survival. Oncology, *57:* 149–156, 1999.

16. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. Methylation of the estrogen receptor CpG island links aging and neoplasia in human colon. Nat. Genet., *7:* 536–540, 1994.

17. Kuismanen, S. A., Holmberg, M. T., Salovaara, R., Schweizer, P., Aaltonen, L. A., de La Chapelle, A., Nystrom-Lahti, M., and Peltomaki, P. Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. Proc. Natl. Acad. Sci. USA, *96:* 12661–12666, 1999.

18. Samowitz, W. S., Slattery, M. L., and Kerber, R. A. Microsatellite instability in human colonic cancer is not a useful clinical indicator of familial colorectal cancer. Gastroenterology, *109:* 1765–1771, 1995.

19. Aaltonen, L. A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., Chadwick, R. B., Kaariainen, H., Eskelinen, M., Jarvinen, H., Mecklin, J. P., and de la Chapelle, A. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N. Engl. J. Med., *338:* 1481–1487, 1998.

20. Chao, A., Gilliland, F., Willman, C., Joste, N., Chen, I. M., Stone, N., Ruschulte, J., Viswanatha, D., Duncan, P., Ming, R., Hoffman, R., Foucar, E., and Key, C. Patient and tumor characteristics of colon cancers with microsatellite instability: a population-based study. Cancer Epidemiol. Biomark. Prev., *9:* 539–544, 2000.

21. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

22. Pao, M. M., Liang, G., Tsai, Y. C., Xiong, Z., Laird, P. W., and Jones, P. A. DNA methylator and mismatch repair phenotypes are not mutually exclusive in colorectal cancer cell lines. Oncogene, *19:* 943–952, 2000.

23. Bender, C. M., Gonzalgo, M. L., Gonzales, F. A., Nguyen, C. T., Robertson, K. D., and Jones, P. A. Roles of cell division and gene transcription in the methylation of CpG islands. Mol. Cell. Biol., *19:* 6690–6698, 1999.

24. Nguyen, T. T., Nguyen, C. T., Gonzales, F. A., Nichols, P. W., Yu, M. C., and Jones, P. A. Analysis of cyclin-dependent kinase inhibitor expression and methylation patterns in human prostate cancers. Prostate, *43:* 233–242, 2000.

Mismatch Repair Deficiency and CpG Island Hypermethylation in Sporadic Colon Adenocarcinomas

Zhenggang Xiong, Anna H. Wu, Christina M. Bender, et al.

Cancer Epidemiol Biomarkers Prev 2001;10:799-803.