The Relationship between Global Methylation Level, Loss of Heterozygosity, and Microsatellite Instability in Sporadic Colorectal Cancer

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Abstract

Purpose: The relationship between global hypomethylation, chromosomal instability (CIN), and microsatellite instability (MSI) remains unclear in colorectal cancer. The aim of this study was to investigate the relationship between global methylation status, loss of heterozygosity (LOH), and MSI in sporadic colorectal cancer.

Experimental Design: We determined global methylation levels in 80 sporadic colorectal cancers, 51 adjacent normal tissues, and 20 normal tissues using the long interspersed nucleotide elements – combined bisulfite restriction analysis method. We also analyzed 80 colorectal cancers for MSI status and LOH at chromosomes 5q21, 8p12-22, 17p13, and 18q21.

Results: We identified 14 cases of MSI (17.5%) and 58 cases of LOH (72.5%). LOH was observed more frequently in microsatellite stable (MSS) cancers than in MSI cancers at all loci. Colorectal cancers showed significantly lower global methylation levels than did normal tissues (41.0 $\pm$ 9.7% versus 54.3 $\pm$ 6.5%; $P < 0.001$). MSS cancers showed significantly lower global methylation levels when compared with MSI cancers (39.5 $\pm$ 9.4% versus 48.2 $\pm$ 8.2%; $P = 0.003$). Tumors with global hypomethylation (with $\leq$40% of methylation levels) had a significantly increased number of chromosomal loci with LOH than did tumors without global hypomethylation (1.9 versus 0.9; $P < 0.001$); 11 tumors (13.9%) lacked both MSI and LOH. This subgroup had significantly higher global methylation levels (46.8 $\pm$ 8.7%) than did MSS cancers with LOH (38.0 $\pm$ 9.0%; $P = 0.006$).

Conclusions: These data showed a significant association between global hypomethylation and chromosomal instability in sporadic colorectal cancer. This suggests that global hypomethylation plays an important role in inducing genomic instability in colorectal carcinogenesis.

Two distinctive pathways of genetic instability, chromosomal instability (CIN) and microsatellite instability (MSI), have been implicated in colorectal carcinogenesis (1). CIN occurs frequently in sporadic colorectal cancer and causes the inactivation of tumor suppressor genes including APC, p53, and SMAD2/SMAD4 (2, 3). Tumors with CIN frequently have gross cytogenetic abnormalities accompanied by allelic losses (1, 4). Chromosomal loss characterized by loss of heterozygosity (LOH) is a predictor of poor survival (5). MSI is caused by the dysfunction of mismatch repair genes including hMLH1 and hMSH2 (6). MSI occurs in 15% to 20% of sporadic colon cancers (7) and most cases of hereditary nonpolyposis colorectal carcinoma (8). However, tumors from patient with sporadic cancer with MSI and hereditary nonpolyposis colorectal carcinoma exhibit considerably different epigenetic characteristics (9).

Both global hypomethylation and region-specific hypermethylation of CpG islands in specific regions of gene promoters are common epigenetic events which take place in the mammalian genome, leading to oncogenesis (10–13). Recent studies have also indicated that global hypomethylation can lead to activation of proto-oncogenes and CIN (14–16), whereas regional hypermethylation leads to the suppression of housekeeping and cell cycle control genes as well as tumor suppressor and DNA repair genes (12, 17). In sporadic colorectal cancer, these two epigenetic alterations have been suggested to contribute to two distinct pathways of carcinogenesis, CIN and MSI (1).

In sporadic colon cancer with MSI, loss of expression of hMLH1 (18) and methylation of CpG sites in a specific region of the hMLH1 promoter (19, 20) are frequently observed. Recent studies have also shown that CpG islands in select sites of the genome are preferentially methylated in tumors. Tumors with these sites methylated have CpG island methylation phenotype, and sporadic colorectal cancers with MSI are frequently found to exhibit the CpG island methylation phenotype (21). Based on these findings, the causal relationship between the CpG island

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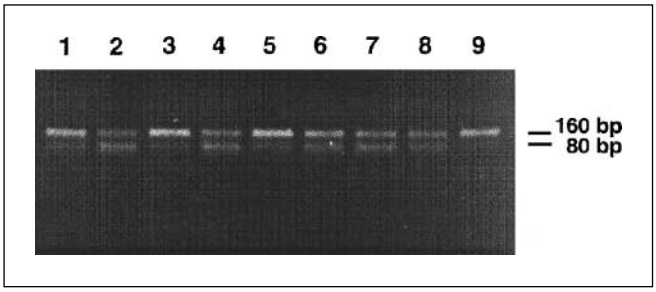

Fig. 1. Assessment of global methylation status by LINE-COBRA. DNA was treated with sodium bisulfite, amplified by PCR with primers LINE3/LINE4, and digested with restriction enzyme TaqI. The global methylation status was calculated by the ratio of the digested band divided by the sum of the digested and undigested bands as described in Materials and Methods. Lanes 1-9, different tumor samples; lanes 1, 3, 5, 6, and 9, tumors with global hypomethylation; lanes 2, 4, 7, and 8, tumors without global hypomethylation.

methylation phenotype and MSI has been suggested. Global hypomethylation has been observed in most cancers including sporadic colorectal cancers and polyps (11, 22, 23). However, the relationship between global hypomethylation, LOH and MSI remains unclear (22, 24).

The purpose of our study was to investigate the relationship between global methylation, CIN and MSI in sporadic colorectal cancer. Our data indicate that a significant correlation exists between global hypomethylation and LOH, suggesting a causal relationship between global hypomethylation and CIN in sporadic colorectal cancer.

Materials and Methods

Patient and tissue samples. Eighty sporadic colorectal cancer tissues and 20 normal colon tissues were obtained from the Department of Pathology at the University of California, San Francisco and the San Francisco VA Medical Center. This study was approved by their respective Institutional Review Boards. All 80 tumors were from patients without a family history of colorectal cancer meeting established criteria for familial adenomatous polyposis or hereditary non–polyposis colorectal cancer.

DNA extraction. Tumors were microdissected from formalin-fixed, paraffin-embedded 7-μm-thick sections stained with H&E using a surgical scalpel under microscopic guidance. Normal colorectal mucosa from 20 noncancer patients and adjacent normal tissues from 80 colorectal cancer patients (51 adjacent normal colorectal mucosa and 29 adjacent muscle tissues when the adjacent colorectal mucosa were not available from the same slide) were also microdissected. Genomic DNA was extracted from microdissected tissue. In brief, microdissected tissue was treated with 100 μL of digestion buffer containing 0.5% Tween 20 (Sigma, St. Louis, MO), 100 mmol/L of Tris, 1 mmol/L of EDTA, and 20 μg of proteinase K (Sigma), and samples were incubated at 56°C overnight. Proteinase K was inactivated by incubating the samples at 95°C for 10 minutes. The extracted DNA was stored at −20°C until ready for use.

Microsatellite instability analysis. MSI was determined by comparing the PCR patterns of tumors with their normal counterparts amplified with the polymorphic loci BAT26, D5S1453, D8S1130, D11S1999, D17S969, D17S1537, and D18S877. Samples showing allelic shift at three or more of seven loci in the tumor compared with adjacent normal tissue were scored as MSI, whereas those showing differences at two or less loci were scored as microsatellite stable (MSS).

Loss of heterozygosity analysis. CIN was determined using LOH events on 5q21, 8pl2-22, 17pl3, and l8q21. LOH analysis was carried out by comparing electrophoresis patterns of tumor and matched normal DNA as described previously (25). DNA of tumors and normal tissues from the same patients were amplified by PCR with four tetranucleotide repeat polymorphic primers in each chromosome. D5S1453, D5S1461, D5S1478, and CHLC.GATA3F03 were in chromosome 5q21 adjacent to the APC gene. The primers D17S1298, D17S1541, D17S1537, and D17S969 are located within 15 cM area on chromosome 17p13 close to the p53 gene. The primers including D18S877, D18S536, D18S846, and CHLC.GATA41El0 are located within a 20 cM region on chromosome 18p21 close to SMAD2/SMAD4 genes. D8S1098, D8S1106, D8S1121, and D8S1130 were used to assess LOH in the 8p12-22 region. The PCR products were separated on a 5% or 6% polyacrylamide gel. The density of each band representing each allele was measured with a densitometer. The ratio of densities of each band representing two alleles in the tumor sample was normalized by the ratio of densities from two alleles in the normal counterpart of the same patient. Tumors with the ratio of either <0.5 or >2.0 were scored as allelic loss. Those showing allelic loss in more than one of four regions were scored positive for LOH.

Assessment of global DNA methylation status. A modified long interspersed nucleotide elements–combined bisulfite restriction analysis (LINE-COBRA) method was used to analyze the global DNA methylation status of cancer and normal mucosa (22, 26, 27). This method is based on the principle that cytidine in DNA is converted to thymidine when DNA is treated with sodium bisulfite, whereas methylated cytidine is protected from the conversion (28). Thus, the methylated and unmethylated cytidine can be distinguished by digestion with a restriction enzyme, which recognizes a sequence containing CpG. Extracted DNA was treated with sodium bisulfite, and isolated by using the EZ DNA methylation kit (Zymo Research, Orange, CA). Bisulfite-treated DNA was amplified by 40 cycles of PCR with two primers, LINE3 (5′-GYGTAAGGGGTTAGGGAGTTTTT) and LINE4 (5′-AACRTAAAACCCTCCRAACCAAATATAAA), with an annealing temperature of 50°C. PCR products were digested with restriction enzyme TaqI, which recognizes TCGA for 1 hour at 65°C, and were separated by electrophoresis on 2% agarose gels. The densities of the digested and undigested bands were obtained by scanning with UMAX Vistascan 3.5.2 and scoring with NIH Image. The ratio of the digested fragment (80 bp) derived from methylated DNA divided by the sum of the digested fragment and undigested fragment (160 bp) derived from unmethylated DNA represents the fractional methylation (expressed as a percentage) at the LINE TaqI site (Fig. 1). Because our unpublished cell line data indicated that ≤40% global methylation levels correctly

Table 1. Clinicopathologic features of MSS and MSI cancer

Features	Total	MSI	MSS	*P*
No. of cases (%)	80	14 (17.5)	66 (82.5)	
Age (y)				
Mean ± SD	68.9 ± 13.0	77.8 ± 8.8	67.4 ± 12.9	0.005
Range	44-90	64-90	44-90	
Gender				
Male	46	8	36	0.37
Female	32	4	28	
Unknown	4	2	2	
Location of tumor				
Right colon	33	10	23	0.002
Left colon	45	2	43	
Unknown	2	2	0	
Dukes stage				
A-B	42	7	35	0.58
C-D	32	5	27	
Unknown	6	2	4	

Table 2. Frequencies of LOH at four loci

Chromosomal loci	Total (n = 80)	MSS (n = 66)	MSI (n = 14)	P (MSS vs. MSI)
5q21 loss	21.3% (17)	24.2% (16)	7.1% (1)	0.16
8p12-22 loss	37.5% (30)	42.4% (28)	14.3% (2)	0.048
17p13 loss	50.0% (40)	60.6% (40)	0% (0)	0.002
18q21 loss	42.5% (34)	51.5% (34)	0% (0)	<0.001

distinguished the cell lines with MSS, CIN, and global hypomethylation from those with MSI, chromosomal stability, and no global hypomethylation, we arbitrarily used ≤40% to define global hypomethylation. Tumor tissues with 40% or lower methylation levels were arbitrarily defined as having global hypomethylation. Those with >40% methylation levels were defined as without global hypomethylation.

Statistical analysis. Comparisons of categorical variables were made using χ^2 test or Fisher's exact test as appropriate. The Mann-Whitney U test was used to compare the global methylation level of each categorical variable. A one-way ANOVA with post hoc test was used to compare the global methylation levels in normal tissues from subjects without cancer, adjacent normal tissues, and cancer tissues. Student's t test was used to compare the mean age of the patients in MSS and MSI cancer.

Results

Microsatellite instability analysis of tumors. Normal colorectal mucosa from the same tumor slide was used as a control for each sample in MSI analysis. If normal colorectal mucosa was not available, we used muscle tissue without cancer cell infiltration from the same tumor slide as a control. Of the 80 colorectal cancers, allelic shifts at three or more of seven loci were observed in 14 cases (17.5%). The remaining 66 cases (82.5%) showing allelic shift of two or less loci were defined as MSS. Clinicopathologic features and MSI and MSS status of all sporadic colorectal cancers are shown in Table 1. The patients with MSI tumors were significantly older than patients with MSS tumors (77.8 ± 8.8 versus 67.4 ± 12.9; P = 0.005). 10 of 14 (71.4%) MSI cancers were located in the right colon, a significantly higher percentage than in MSS cancers (34.8%; P = 0.002). There was no significant difference between the MSI and MSS groups with respect to gender (P = 0.23) or Dukes stage (P = 0.37). For patients with MSI tumors, we did not observe MSI in all 51 adjacent normal tissues we tested. This was consistent with our previous observation that when the immunostaining of tumors by hMLH1 and hMSH2 antibodies was negative, the immunostaining of adjacent normal tissues was positive as was the distal normal tissues.

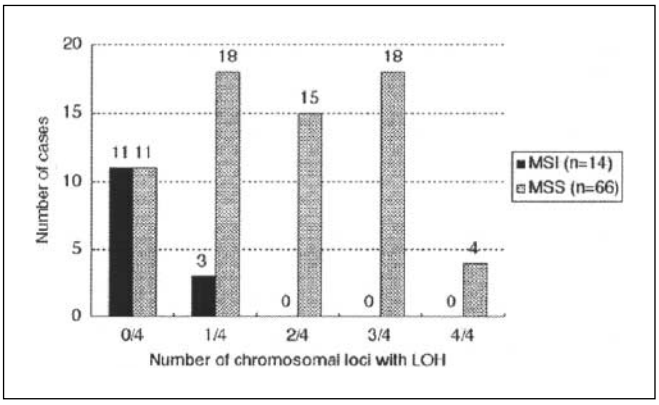

Fig. 2. Comparison of the number of chromosomal loci with LOH in MSI (n = 14) and MSS cancers (n = 66). The number of chromosomal loci with LOH in MSS cancers was significantly larger than that in MSI cancers (P < 0.001).

Table 3. DNA methylation levels in sporadic colorectal cancer

	n	Methylation level (%)	P
Normal tissues	20	54.3 ± 6.5	
Adjacent normal	51	46.2 ± 8.0	0.003*
All cancer	80	41.0 ± 9.7	<0.001*, 0.006†
Age (y)			
<65	32	39.4 ± 10.9	0.47
>65	42	41.8 ± 9.4	
Gender			
Male	44	40.2 ± 9.9	0.52
Female	32	41.9 ± 10.2	
Location			
Right	33	44.2 ± 8.8	0.02
Left	45	38.4 ± 10.0	
Dukes stage			
A-B	42	41.4 ± 10.1	0.49
C-D	32	39.8 ± 9.7	
Microsatellite status			
MSI	14	48.2 ± 8.2	0.003
MSS	66	39.5 ± 9.4	
In MSS cancer			
5qLOH(+)	16	35.3 ± 9.0	0.07
(−)	50	41.0 ± 9.4	
8pLOH(+)	28	37.5 ± 9.0	0.20
(−)	38	41.2 ± 9.8	
17pLOH(+)	40	37.5 ± 10.0	0.03
(−)	26	42.8 ± 8.0	
18qLOH(+)	34	36.2 ± 9.4	0.006
(−)	32	43.3 ± 8.4	

*Compared with normal tissues.
† Compared with adjacent normal tissues.

Loss of heterozygosity analysis. We used four polymorphic loci with a high percentage of heterozygosity for LOH analysis in each chromosome region. In this study, all normal tissues (either colorectal mucosa or muscle tissues) from the 80 colorectal cancer patients were informative for LOH at each chromosomal locus. We identified 58 cancers (72.5%) with LOH at one or more of four chromosomal regions. Twenty-two cancers (11 cases with MSI and 11 cases with MSS) had no evidence of chromosomal loss. The highest frequency of LOH was found at the p53 locus on 17p13 (40 cases, 50.0%), followed by the SMAD2/SMAD4 region on 18q21 (34 cases, 42.5%), 8p12-22 (30 cases, 37.5%), and APC locus on 5q21 (17 cases, 21.3%; Table 2). MSS cancers showed significantly higher rates of LOH on three of four chromosomal loci than did MSI cancers (24.2% versus 7.1%; P = 0.16 in 5q21, 42.4%

Table 4. Relationship between global methylation levels and number of chromosomal loci with LOH

Global methylation levels	No. of cases	No. of chromosomal loci with LOH					*P*
		0 of 4	1 of 4	2 of 4	3 of 4	4 of 4	
All cancers	80	22	21	15	18	4	
≤40%	50	9 (41%)	11 (52%)	11 (73%)	15 (83%)	4 (100%)	<0.001*
				41 (71%)			
>40%	30	13 (59%)	10 (48%)	4 (27%)	3 (17%)	0 (0%)	
				17 (29%)			

*Compared with tumors with >40% global methylation.

versus 14.3%; $P = 0.048$ in 8p12-22; 60.6% versus 0%; $P = 0.002$ in 17p13, and 51.5% versus 0%; $P < 0.001$ in 18q21). Figure 2 shows the number of chromosomal loci with LOH in MSI versus MSS cancers. More MSS tumors (37 of 66, 56.1%) showed LOH at two or more loci and the mean number of chromosomal loci containing LOH in MSS cancers was significantly greater than that in MSI cancers (1.8 versus 0.2, $P < 0.001$). Loss of individual chromosomal loci showed no significant correlation with age, gender, tumor location, and Dukes stage (data not shown).

Assessment of global DNA methylation level. Global DNA methylation levels in 80 colorectal cancers, 51 adjacent normal colorectal mucosa from cancer patients, and 20 normal colorectal mucosa from subjects without cancer was examined by using the modified LINE-COBRA method (Fig. 1; Table 3). Normal tissues were highly methylated (54.3 ± 6.5%), whereas adjacent normal tissues had significantly lower methylation levels than did normal tissues (46.2 ± 8.0%, $P = 0.003$). Cancer tissues had significantly lower global methylation levels (41.0 ± 9.7%) than did normal tissues or adjacent normal tissues. ($P < 0.001$, $P = 0.006$, respectively). Because the methylation levels may change with age, we need to consider this factor in comparing methylation levels between different tissues. The age of 20 noncancer patients carrying normal tissues (67.8 ± 12.6) is matched with that of 80 colorectal cancer patients (68.9 ± 13.0). The 51 adjacent normal tissues were randomly selected from the 80 colorectal cancer patients depending on the availability of the normal appearing tissues on the same slide. Thus, these differences between tumors and normal tissues or adjacent normal tissues may not be biased by age. Because the methylation pattern might be tissue-specific, muscle tissues are not appropriate to be used for comparison with mucosa or colorectal cancer in methylation analysis. Thus, we did not include the 29 adjacent muscle tissues in global methylation analysis. Colorectal cancers with MSS showed significantly lower global methylation levels than MSI tumors (39.5 ± 9.4% versus 48.2 ± 8.2%; $P = 0.003$). MSS tumors had significantly lower methylation levels than their adjacent normal counterparts (39.5 ± 9.4% versus 45.3 ± 6.5%; $P = 0.01$), whereas MSI tumors did not show lower methylation levels than the adjacent normal tissues (48.2 ± 8.2% versus 47.8 ± 7.5%; $P = 0.70$). Tumors located in the left colon had a significantly lower global methylation level than right colon tumors (38.4 ± 10.0 versus 44.2 ± 8.8; $P = 0.02$). No significant difference was observed with respect to age, gender, and Dukes stage. In tumors with MSS, LOH-positive cancers showed lower methylation levels compared with LOH-negative cancers (in chromosomal loci 5q, $P = 0.07$; 8p, $P = 0.2$; 17p, $P = 0.03$; and 18q, $P = 0.006$). Table 4 shows the relationship between levels of global methylation and the number of chromosomal loci with LOH. Tumors with global hypomethylation had dramatically increased levels of LOH than those without global hypomethylation (1.9 versus 0.9 LOH loci; $P < 0.001$), suggesting a strong correlation between global hypomethylation and CIN.

The relationship between microsatellite status and chromosomal instability. We classified all cancers into four groups according to microsatellite status and CIN status as indicated by the presence of LOH. MSS cancers with LOH were the most

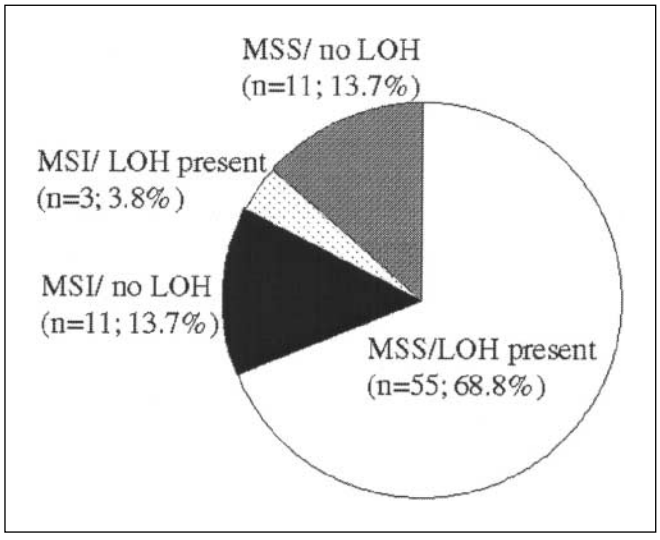

Fig. 3. Genomic instability patterns classified by microsatellite status and CIN of 80 colorectal cancers. MSS cancers with LOH were most common (55 cases, 69.9%), followed by MSI cancers without LOH (11 cases, 13.9%), MSS cancers without LOH (11 cases, 13.9%), and MSI cancers with LOH (3 cases, 3.8%).

Table 5. Comparison between MSS/no LOH tumors and MSS/LOH tumors

Features	MSS/no LOH	MSS/LOH	*P*
No. of cases	11	55	
Age (y)	65.4 ± 16.7	67.8 ± 12.1	0.29
Gender			
Male	6	30	0.68
Female	5	23	
Location of tumor			
Right colon	5	18	0.32
Left colon	6	37	
Dukes stage			
A-B	7	28	0.28
C-D	3	24	
Unknown	1	3	
Global methylation levels			
Mean ± SD (%)	46.8 ± 8.7	38.0 ± 9.0	0.006
≤40%	4	41	0.02
>40%	7	14	

common (55 cases, 68.8%), followed by MSI cancers without LOH (11 cases, 13.7%), and MSS cancers without LOH (11 cases, 13.7%). Only three cases (3.8%) had MSI cancers with LOH (Fig. 3). The rare occurrence of cases with both MSI and LOH could be explained by the close correlation between MSI and no LOH or between MSS and LOH. Interestingly, nearly 14% of sporadic colorectal cancers lacked either MSI or LOH. We compared the clinicopathologic features and global methylation levels of patients with tumors with MSS without LOH with those of MSS cancers with LOH (Table 5). A significantly lower percentage of MSS cancers without LOH showed global hypomethylation compared with MSS cancers with LOH (4 of 11 cases, 36.4%; versus 41 of 55 cases, 74.5%; $P = 0.02$). Moreover, the level of global methylation was significantly higher in MSS cancers without LOH (46.8 ± 8.7%) than in MSS cancers with LOH (38.0 ± 9.0; $P = 0.006$). No statistical difference was observed between the two groups with respect to age, gender, tumor location, and Dukes stage (Table 5).

Discussion

In this study of sporadic colorectal cancer, MSI was found in 17.5% of all cases and was associated with distinct clinicopathologic features including older age and right-sided location. These results are consistent with the previous reports (29–31). We also used LOH in four chromosomal regions as evidence for CIN. The regions studied included those linked to common tumor suppressor genes in colorectal cancer including APC, p53, and SMAD2/SMAD4. High frequency of LOH in these regions has been reported previously (5, 32). In our study, a high frequency of LOH was observed in cancers with MSS at four loci: 17p13 (60.6%), 18q21 (51.5%), 8p12-21 (42.4%), and 5q21 (24.2%). By contrast, in MSI cancers, we found a lower frequency of LOH. Also, the extent of CIN was lower in MSI cancers, as judged by the number of chromosomal loci exhibiting LOH. These findings are consistent with previous studies (30, 32, 33) and suggest that colorectal cancer with MSI and MSS develop differently.

We applied a modified LINE-COBRA PCR method to efficiently and quantitatively analyze LINE sequence methylation status. This protocol targets shorter amplicon sizes of the widely distributed LINE sequences, which greatly improve the yield when amplifying genetic material derived from microdissected paraffin-embedded tissue (27). Global hypomethylation has been detected in colonic hyperplastic polyps, adenoma, and colorectal cancer (22, 23). Suter et al. have reported that adjacent normal mucosa from cancer patients exhibited lower level of genomic methylation than the mucosa from healthy individuals (34). We also observed that colorectal cancers and adjacent normal tissues showed significantly lower global methylation levels than did normal tissues from the subjects without cancer. Moreover, we found that cancers with MSS showed significantly lower global methylation levels than those with MSI. To our knowledge, this has not been previously reported. These findings suggest that colorectal cancers with MSI and MSS may develop through two distinct epigenetic pathways, regional hypermethylation (CpG island methylation phenotype) and global hypomethylation (23, 35).

In global methylation analysis, we showed that LOH-positive cancers have lower global methylation levels compared with LOH-negative cancers. We also found that tumors with global hypomethylation showed a significantly higher number of chromosomal loci with LOH. The reasons by which global hypomethylation may cause LOH are not clear (12, 13). Eden et al. have shown that DNA hypomethylation increased the rate of LOH in mouse embryonic fibroblasts (14). Recently, it has also been reported that DNA CpG hypomethylation induces a significant reorganization of constitutive pericentric heterochromatin (36). Our observation that LOH significantly correlated with global hypomethylation might be explained by the possibility that global hypomethylation may affect the molecular composition of heterochromatin and chromatin conformation, and may promote LOH by destabilizing the genome.

We classified the cancers in this study into four groups by microsatellite and LOH status. The genomic instability patterns of the tumors in our study are very similar to that of the recent report by Goel et al. (33). The most common group was MSS with LOH (69.9%) followed by MSI cancers without LOH (13.9%). We also identified nearly 14% of sporadic colorectal cancers that lacked LOH or MSI. This subgroup of tumors with MSS without LOH showed significantly higher global methylation levels than MSS cancers with LOH. The pathogenic pathways leading to tumorigenesis in this subgroup is not clear. It is possible that these tumors have LOH in other chromosome regions or that aberrant promoter methylation may cause silencing of other tumor suppressor genes (21). It is also possible that other novel pathways may be involved in the pathogenesis of this subgroup of colorectal cancer.

In conclusion, our study shows that the global hypomethylation status of colorectal tumors correlates with LOH. These results, together with recent studies by other investigators (12–16), suggest that global hypomethylation predisposes to CIN, and therefore plays an important role in colorectal carcinogenesis.

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