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Proximal and distal colorectal cancers show distinct gene-specific methylation profiles and clinical and molecular characteristics

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ABSTRACT

Introduction: Accumulation of genetic and epigenetic alterations contribute to malignant transformation of normal colonic mucosa to cancer. However, the frequency and the pattern of these alterations in proximal and distal colon cancers have not been examined in detail.

Methods: In this study, we examined methylation frequencies and patterns using 14 marker genes in 31 proximal and 43 distal colorectal cancers. We also analysed the relationship between these parameters and clinical characteristics, MSI, mutations of BRAF, KRAS and p53, LOH and global hypomethylation.

Results: Three groups of tumours with varying degrees of methylation frequencies were identified: very high (9%), high (22%) and low (69%) methylation. Tumours with very high and high methylation showed more frequent proximal location, MSI, *BRAF* mutations and less frequent LOH and global hypomethylation. The methylation markers could be classified into 3 types based on methylation frequencies, MSI status and location. Proximal tumours showed more frequent methylation of Type 2 markers, CIMP+, MSI, *BRAF* mutations and lower frequencies of LOH and global hypomethylation, whilst Type 3 marker, *MGMT* methylation was more frequently associated with distal tumours, better survival and G to A mutation in non-CpG sites in *KRAS* and *p53* genes.

Conclusion: These data showed that proximal and distal colorectal cancers have distinct gene-specific methylation profiles and molecular and clinical characteristics.

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1. Introduction

In colorectal carcinogenesis, progressive accumulation of genetic and epigenetic alterations contributes to malignant transformation of normal colonic mucosa to cancer.[1,2] Chromosomal and microsatellite instability pathways constitute major genetic instability events in colorectal cancer.[2,3] Epigenetic changes include CpG island promoter methylation and global hypomethylation, both of which have been reported to occur early in colorectal carcinogenesis.[4–8] Promoter

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Table 1 – Primers for CpG island methylation analysis

Gene or locus		Forward primer (5'–3')/reverse primer (5'–3')	Size (bp)	T_m (°C)
hMLH1	Met	AGCGGATAGCGATTTTTAACGC/ ACTCTATAAATTACTAAATCTCTTCG	98	59
	Unmet	GAAGAGTGGATAGTGATTTTTAATGT/ ACTCTATAAATTACTAAATCTCTTCA	103	57
p16ink4A	Met	TAGAGGGTGGGGCGGATCGC/ CATACTACTCCCCGCCGCCG	103	68
	Unmet	ATTAGAGGGTGGGGTGGATTGT/ CCATACTACTCCCCACCACCA	106	64
HlC1	Met	AGGCGTCGGGTTCGCGC/ CCCGAACGAACACGACG	104	64
	Unmet	AGGGAGGTGTTGGGTTTGTGT/ CAACACCCCAAACAAACACAACA	114	62
RASSF2	Met	ATTTTACGGTTTTTGCGGCGC/ TTACGCCGAACATCCCGCG	116	62
	Unmet	TTAATTTTATGGTTTTTGTGGTGTGGGTGT/ TACACCTTACACCAAACATCCCACA	125	62
MINT1	Met	TAAATTTTTTTATATATATTTTCGAAGC/ ACAAAAAACCTCAACCCCGCG	101	58
	Unmet	TTTAAATTTTTTTATATATATTTTTGAAGT/ CTAACAAAAAAACCTCAACCCCACA	106	56
MINT31	Met	TGTTGGGGAAGTGTTTTTCGGC/ CCCGAAAACGAAACGCCGCG	86	65
	Unmet	GATGTTGGGGAAGTGTTTTTTGGT/ AAATACCCAAAAACAAAACACCACA	93	60
SFRP1	Met	TTCGGTCGTAGGAGTTTCGC/ CGACTCCCGAAAATACGACG	103	62
	Unmet	AGGGTTTGGTTGTAGGAGTTTTGT/ CACCCCAACTCCCAAAAATACAACA	112	62
SFRP2	Met	CGGAGTTTTTCGGAGTTGCGC/ ATTCGAACTTATCCCGAACCCG	153	64
	Unmet	TGGGTTGGAGTTTTTTGGAGTTGTGT/ CCAAAATTCAAACTTATCCCAAACCCA	163	62
SFRP4	Met	TGTAGTTGTTAAGGGTGCGTTTC/ ACGCCAACTCTCAACCTTCG	155	62
	Unmet	TGGTTGTAGTTGTTAAGGGAGTGTTTT/ AACAACACCAACTCTCAACCTTCA	163	61
SFRP5	Met	CGTAAGATTTGGCGTTGGGC/ CCCAACCAATCTCCGACCG	113	62
	Unmet	GGGTGTAAGATTTGGTGTTGGGT/ CACCCCAACCAATCTCCAACCA	119	64
SLC5A8	Met	GGGTAGCGGGTCGATTTTTC/ CGAACGCACCCCGAAACG	110	63
	Unmet	GTATTTAGGGTAGTGGGTTGATTTTTT/ CTCCAAACACACCCCAAAACA	120	60
TAC1	Met	TATTGAGTAGGCGAAAGAGCGC/ TCTAATTCCTCCGAACGCACG	92	63
	Unmet	TAAGGTATTGAGTAGGTGAAAGAGTGT/ CTAAATTTCTCTAATTCCTCCAAACACACA	106	62
SST	Met	GGGGGCGTTTTTTAGTTTGAC/ AACAACGATAACTCCGAACCTCG	104	62
	Unmet	GAGATTTGGGGGTGTTTTTTAGTTTGAT/ CAACAAACAACAACAATAACTCCAAACCTCA	119	62
MGMT	Met	GTAGGTCGTTTGTACGTTCGC/ CGACCGATACAAACCGAACG	122	62
	Unmet	GTATGTGGTAGGTTGTTTGTATGTTTGT/ ACCCTTCAACCAATACAAACCAAACA	135	62

hypermethylation has been shown to lead to transcriptional silencing of various genes such as tumour suppressor genes and genes involved in cell cycle control, DNA repair and apoptosis, whereas global hypomethylation can lead to chromosomal instability.[4–8] Two types of promoter methylation have been described, age-related and cancer specific.[9] Many gene promoters such as *ER-alpha, EGFR, IFG2* and *MYOD1* have been reported to be methylated in colonic mucosa as a function of age and undergo more extensive methylation in cancer and termed age-related or type A methylation.[10–12] In addition, cancer specific or type C methylation loci such as *p16*, MINT1, MINT2, MINT31 and *MLH1* have also been identified in a subset of tumours.[9,13] When a large number of colorectal cancers were examined, some were found to accumulate high frequencies of type C methylation of multiple genes and this subset of tumours was classified as having CpG island methylator phenotype (CIMP). Subsequent studies have shown that CIMP+ tumours have distinct clinicopathological and molecular characteristics such as older age at diagnosis, proximal location, mucinous histology, high incidence of BRAF mutations, MSI and lower rates of p53 mutations. However, there have been conflicting reports regarding the validity of the existence of CIMP. Some thought that CIMP was primarily related either to ageing, *hMLH1* methylation or MSI.[14,15]

Accumulating evidence suggests that proximal and distal colorectal cancers differ not only in incidence according to geographic location, age and gender but also in molecular biological characteristics. About 60% of colorectal cancers in high incidence areas are found in the distal colon, whilst there is a predominance of proximal tumours in low incidence areas.[16] Proximal tumours occur predominantly in older individuals and in females.[17] The incidence of MSI in proximal tumours has been reported to be higher than

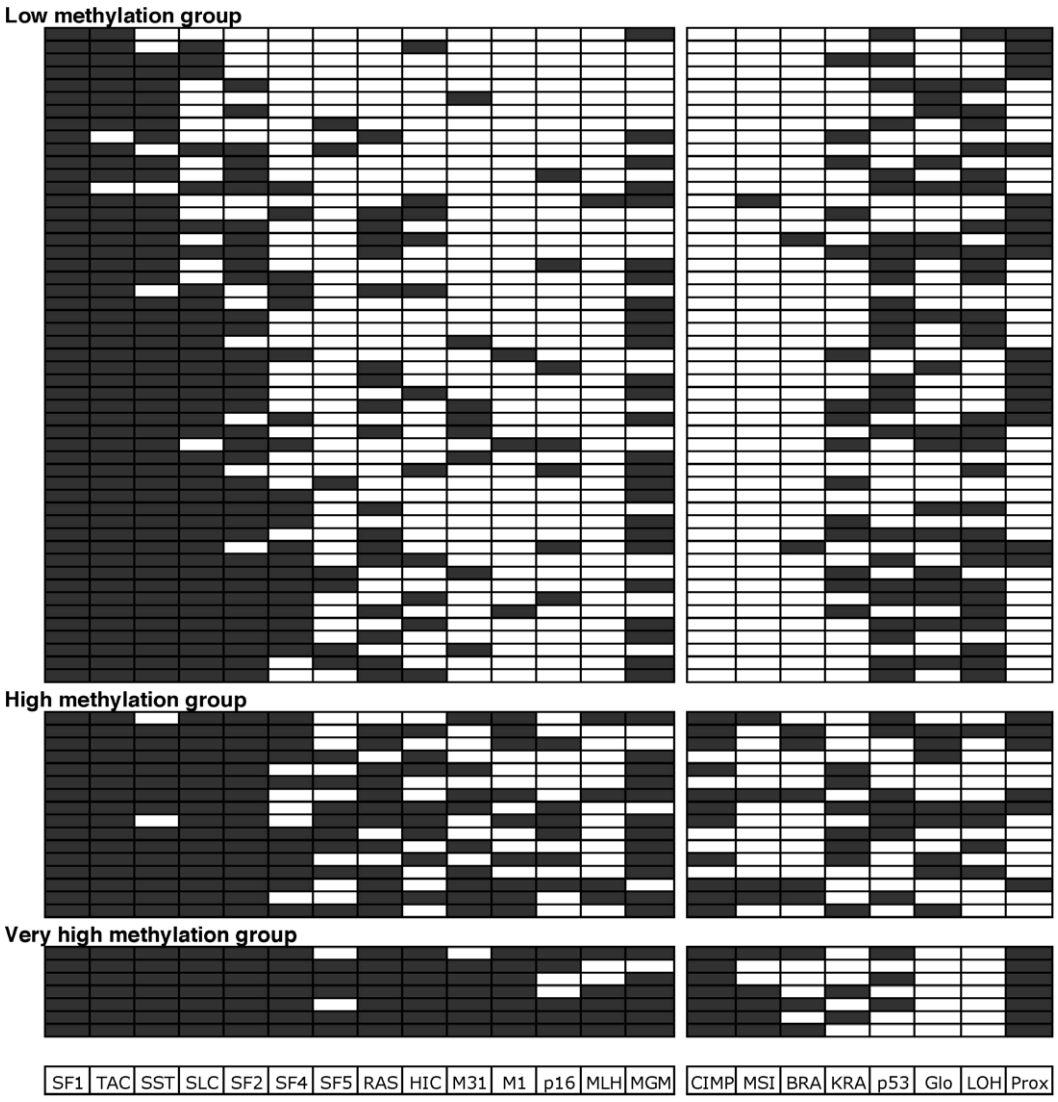

Fig. 1 – Methylation status at 14 loci and clinical and molecular characteristics in colorectal cancers. Each column represents the methylation status of *SFRP1*, *TAC1*, *SST*, *SLC5A8*, *SFRP2*, *SFRP4*, *SFRP5*, *RASSF2*, *HIC1*, MINT31, MINT1, *p16ink4A*, *hMLH1* and *MGMT*, respectively, or CIMP status (methylation at ⩾3/6 type C loci) , MSI, *BRAF*, *KRAS* or *p53* mutations, global hypomethylation, LOH (deletion at ⩾2 of four chromosome regions), and proximal location. Dark box indicates positive results. Horizontal rows represent 74 individual tumours subgrouped as low, high and very high methylation groups.

in distal tumours with up to 10-fold higher incidence observed.[18]

In the present study, we evaluated the frequency and patterns of methylation using 14 marker genes consisting of 6 frequently used type C loci and eight additional loci recently identified as tumour associated in sporadic colorectal cancers and examined their relationship to clinical and molecular features of the tumours. We also compared the clinical and molecular features of CIMP+ tumours defined by 6 traditional type C marker loci to those of CIMP+ tumours defined using 14 loci. Finally, we compared the methylation frequencies and patterns, and other clinical and molecular characteristics of proximal and distal colorectal cancers.

2. Materials and methods

2.1. *Primary colorectal tumours*

Seventy-four archival pathological specimens of colorectal carcinomas were obtained at University of California at San Francisco and San Francisco Veteran Affairs Medical Center. All tumours were from patients with no known family history of colorectal cancers. This study was approved by the Institutional Review Board. Tumour samples were microdissected from formalin-fixed, paraffin-embedded sections stained with haematoxylin–eosin as described previously.[19] Histopathological normal mucosa samples were also microdissected from

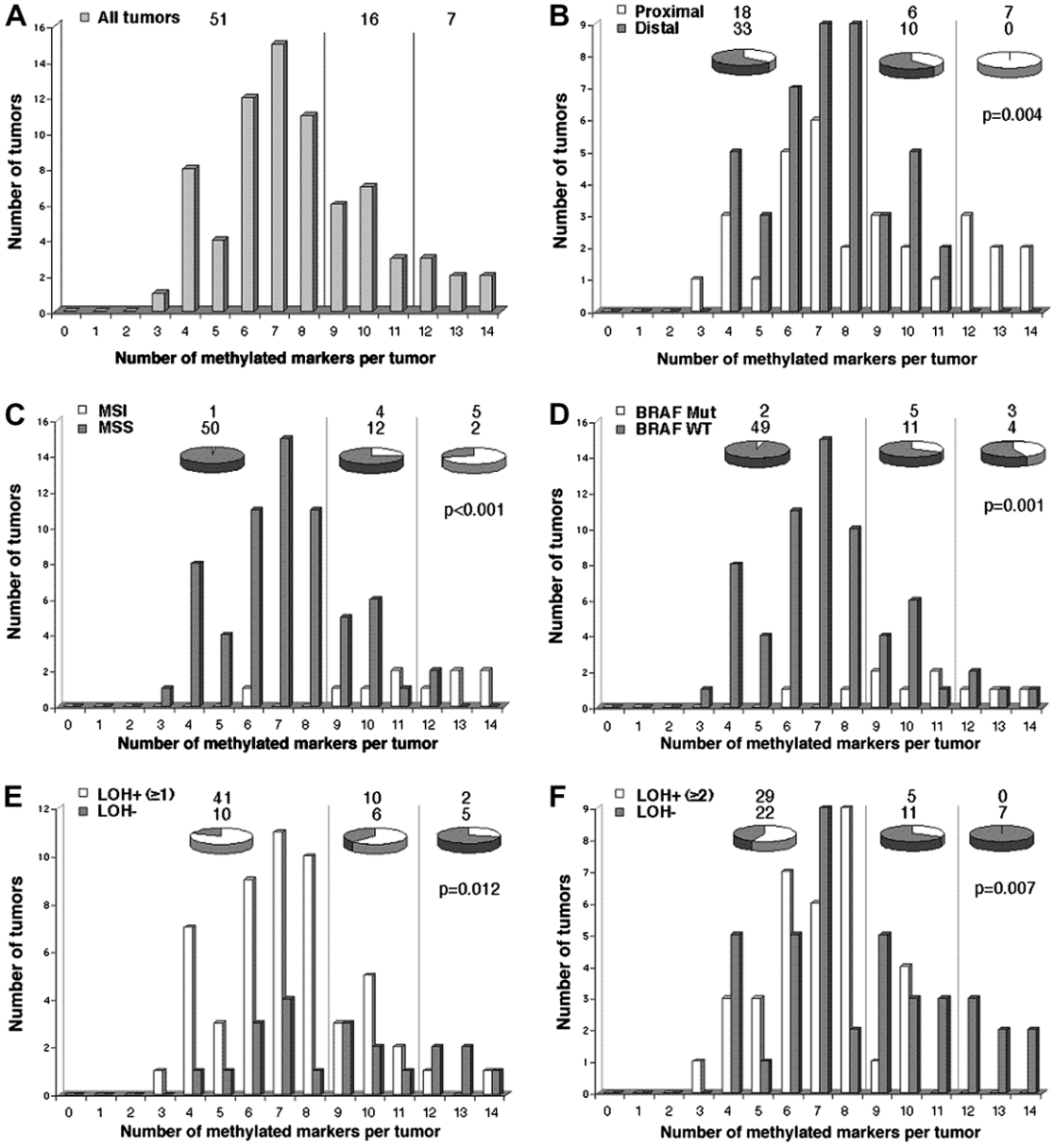

Fig. 2 – Correlation of methylation frequency with clinical and molecular features in colorectal cancer. (A) Each column represents the number of tumours showing different methylation frequencies. Tumours with methylation at eight or less loci, at 9–11 loci and at 12 or more loci were defined as low (51, 69%), high (16, 22%) and very high (7, 9%) methylation groups, respectively. (B–F) Correlation of methylation status with tumour location, MSI, *BRAF* mutations, LOH (⩾1 of four chromosome regions), and LOH (⩾2 of four chromosome regions), respectively.

normal blocks of the same patients taken at least 5 cm away from the tumours.

2.2. DNA methylation analysis in CpG islands

The status of DNA methylation was examined in 14 loci including six frequently used type C loci (*hMLH1*, *p16ink4A*, *HIC1*, *RASSF2*, MINT1 and MINT31) [9,13] and eight additional tumour associated loci (*SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*,[20,21] *SLC5A8*,[22] *TAC1*, *SST*[23] and *MGMT*[24]) by methylation-specific PCR (MSP) as described previously.[25,26] Bisulphite-modified DNA templates were amplified by PCR using either methylation-specific primers or unmethylation-specific primers of these 14 loci (Table 1). CpG island methylation phenotype (CIMP+) was arbitrarily defined by three methods, methylation at $\geqslant$3/6 type C loci, $\geqslant$9/14 loci or $\geqslant$12/14 loci.

2.3. LOH analysis

LOH was analysed in the following chromosome regions: 5q21 (*APC* gene), 8p11-22, 17p13 (*p53* genes) and 18q21 (*DCC/SMAD4*), where LOH was frequently present in colorectal cancers. Four to five polymorphic loci covering around 20 cM in each chromosome region were used, such as D5S1461, D5S1453, D5S1466, D5S1468, D5S1478 (5q21); D8S1130, D8S1106, D8S1121, D8S255, D8S1098 (8p11-22); D17S1298, S17S1537, D17S1541, D17S1303 (17p13); D18S877, D18S536, D18S846 and D18S858 (18q21). LOH status was defined as positive low: $\geqslant$1 of four chromosome regions, or positive high: $\geqslant$2 of four chromosome regions.

2.4. Mutation analysis of BRAF, KRAS and p53 genes

Genomic DNA of tumours was amplified by PCR using primers of the exon 15 of *BRAF* gene (5′-CTTTACTTACTACACCTCAG and 5′-TAACTCAGCAGCATCTCAGG), the exon 1 of *KRAS* gene (5'-ACCTTATGTGTGACATGTTCTAATATAG and 5'-GAATGGTCCT GCACCAGTAA), the exon 5 (5'-GTCTCCTTCCTCTTCCTACAG-TAC and 5'-TCTCTCCAGCCCCAGCTGCT), exon 6 (5'-CCCAG-GCCTCTGATTCCTCA and 5'-CAGAGACCCCAGTTGCAAACCA), exon 7 (5'-AGGCGCACTGGCCTCATCTT and 5'-AGGGTGGCAA-GTGGCTCCT) and exon 8 (5'-CCTCTTGCTTCTCTTTTCCT ATCCTGA and 5'-ACCGCTTCTTGTCCTGCTTGCT) of *p53* gene. The PCR products were sequenced with an ABI PRISM 3100 automated sequencer.

2.5. Global methylation analysis

Global methylation was determined by the combined bisulphite-restriction assay (COBRA), using primers for long interspersed element 1 (LINE1) repetitive sequence.[27] The NaHSO$_3$-modified DNA was amplified by 35 cycles of PCR with annealing at 50 °C. The product was digested with restriction enzyme TaqI, and separated on agarose gel. The ratio of the digested fragment (methylated DNA) over the sum of the digested fragment and undigested fragment (unmethylated DNA) represented the percent of global methylation. Sample with 40% or lower global methylation level was arbitrarily defined as global hypomethylation.

2.6. Statistical analysis

Comparison of categorical variables was made using X^2 test and Fisher exact test. Student's *t*-test was used for comparison of the mean age of the patients. For all of the analyses, $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Relationship between methylation frequency of CpG islands of 14 genes and clinical and molecular features

In defining CpG island methylator phenotype (CIMP) to classify a subset of tumours with high frequency of methylation

Table 2 – Comparison of clinical and molecular characteristics amongst tumours with different CpG island methylation frequencies

	Low methylation (%)	High methylation (%)	Very high methylation (%)	*p*
Number of cases	51	16	7	
Gender				
M	32 (64)	8 (53)	4 (57)	ns
F	18 (36)	7 (47)	3 (43)	
Age	66.6 ± 14.4	71.9 ± 9.3	72.0 ± 13.2	ns
Stage				
A/B	28 (55)	11 (69)	3 (43)	ns
C/D	23 (45)	5 (31)	4 (57)	
Survival (5-year)				
+	28 (55)	9 (56)	1 (14)	ns
–	23 (45)	7 (44)	6 (86)	
Location				
Proximal	18 (35)	6 (38)	7 (100)	0.004
Distal	33 (65)	10 (62)	0 (0)	
MSI				
+	1 (2)	4 (25)	5 (71)	<0.001
–	50 (98)	12 (75)	2 (29)	
BRAF mutation				
+	2 (4)	5 (31)	3 (43)	0.001
–	49 (96)	11 (69)	4 (57)	
KRAS mutation				
+	15 (29)	7 (43)	2 (29)	ns
–	36 (71)	9 (57)	5 (71)	
p53 mutation				
+	26 (51)	7 (43)	3 (43)	ns
–	25 (49)	9 (57)	4 (57)	
LOH ($\geqslant$1)				
+	41 (80)	10 (62)	2 (29)	0.012
–	10 (20)	6 (38)	5 (71)	
LOH ($\geqslant$2)				
+	29 (57)	5 (31)	0 (0)	0.007
–	22 (43)	11 (69)	7 (100)	
Global hypomethylation				
+	18 (35)	8 (50)	0 (0)	0.062
–	33 (65)	8 (50)	7 (100)	

(CIMP+) of multiple genes, 'type C' methylation markers have frequently been used.[9,13] In the present study, we investigated the methylation status of tumours using both traditional 'type C' methylation markers (such as *hMLH1*, *p16ink4A*, *HIC1*, *RASSF2*, MINT1 and MINT31) and additional methylation markers with high methylation frequencies in colorectal cancer, such as *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*,[20,21] *SLC5A8*,[22] *TAC1*, *SST*[23] and *MGMT*[24] genes. We first tested the methylation status of these additional markers, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *SLC5A8*, *TAC1*, *SST* and *MGMT* genes in 21 normal colorectal mucosa from non-cancer patients, and observed no methylation in these markers, indicating that they are tumour specific. The frequency of methylation at these 14 loci in 74 primary tumours was as follows: *SFRP1* (100%), *TAC1* (97%), *SST* (91%), *SLC5A8* (81%), *SFRP2* (80%), *MGMT* (59%), *SFRP4* (51%), *SFRP5* (27%), *hMLH1* (14%), *p16ink4A* (24%), *HIC1* (35%), *RASSF2* (50%), MINT1 (26%) and MINT31 (31%), respectively (Fig. 1). All tumours showed methylation in three or more loci (Fig. 2A). We arbitrarily classified tumours into three groups depending on the numbers of methylated loci per tumour: the tumours with methylation of 8 or less loci were classified as low methylation group, whilst those with methylation of 9–11 loci and those with methylation of 12–14 loci were classified as high and very high methylation groups, respectively (Fig. 2A). We observed that three groups of tumours with different levels of methylation showed significant differences in the location, and in the frequencies of MSI, BRAF mutations and LOH at chromosomes 5q, 8p, 17p and 18q (Fig. 2B–F). All very high methylation group tumours (7/7, 100%) were located in the proximal colon, whereas high (10/16, 63%) and low (33/51, 65%) methylation group tumours were more frequently located in the distal colon. Very high methylation groups of tumours showed the highest frequency of MSI (71%), and BRAF mutation (43%), and the lowest frequency of LOH either at one or more (29%) or two or more chromosomal loci (0%), respectively. By contrast, the low methylation group of tumours showed the lowest frequency of MSI (2%) and *BRAF* mutation (4%), and highest frequency of LOH at either one or more or two or more chromosomes (80% and 57%), respectively. High methylation group tumours showed intermediate frequency of MSI (25%, $p < 0.001$) *BRAF* mutation (31%, $p = 0.001$) and LOH (62%, $p = 0.012$, or 31%, $p = 0.007$), respectively (Fig. 2C–F) (Table 2). There were no significant differences in genders, ages at diagnosis, stages or 5-year survival rates, *KRAS* and *p53* mutations amongst three groups of tumours (Table 2). Overall, in these tumours methylation frequency tended to show inverse correlation with global hypomethylation. None of the seven tumours with very high methylation had global hypomethylation. However, 50% of high methylation and 35% of low methylation group tumours showed global hypomethylation.

In order to gain some insight into the characteristics of CIMP, we arbitrarily defined CIMP according to the methylation frequency of 14 loci or 6 traditional 'type C' loci. The

Table 3 – Comparison of tumour features with CIMP defined by traditional markers with high and very high CIMP defined by 14 markers

	Total	CIMP (⩾3/6)		CIMP (⩾9/14)		CIMP (⩾12/14)	
		− (%)	+ (%)	− (%)	+ (%)	− (%)	+ (%)
Number of cases	74	56	18	51	23	67	7
Location							
Proximal	31	18 (32)	13 (72)	18 (35)	13 (57)	24 (36)	7 (100)
Distal	43	38 (68)	5 (28)	33 (65)	10 (43)	43 (64)	0 (0)
p			0.005		ns		0.001
MSI							
+	10	1 (2)	9 (50)	1 (2)	9 (39)	5 (7)	5 (71)
−	64	55 (98)	9 (50)	50 (98)	14 (61)	62 (93)	2 (29)
p			<0.001		<0.001		<0.001
BRAF mutation							
+	10	2 (4)	8 (44)	2 (4)	8 (35)	7 (10)	3 (43)
−	64	54 (96)	10 (56)	49 (96)	15 (65)	60 (90)	4 (57)
p			<0.001		0.001		0.048
LOH (⩾1)							
+	53	43 (77)	10 (56)	41 (80)	12 (52)	51 (76)	2 (29)
−	21	13 (23)	8 (44)	10 (20)	11 (48)	16 (24)	5 (71)
p			ns		0.024		0.017
LOH (⩾2)							
+	34	31 (55)	3 (17)	29 (57)	5 (22)	34 (51)	0 (0)
−	40	25 (45)	15 (83)	22 (43)	18 (78)	33 (49)	7 (100)
p			0.006		0.006		0.013
Global hypomethylation							
+	26	20 (36)	6 (33)	18 (35)	8 (35)	26 (39)	0 (0)
−	48	36 (64)	12 (67)	33 (65)	15 (65)	41 (61)	7 (100)
p			ns		ns		0.047

tumours with methylation at 3 or more of 6 'type C' loci (18/74, 24%), or at 9 or more of 14 loci (23/74, 31%) or at 12 or more of 14 loci (7/74, 9%) were defined as CIMP+. All tumours with CIMP+ defined by three different methods showed similar molecular characteristics such as significantly higher frequency of MSI, *BRAF* mutations and lower frequency of LOH except for global hypomethylation (Table 3). None of the tumours defined as CIMP+ with methylation at 12 or more of 14 loci (0/7, 0%) showed global hypomethylation compared to CIMP-tumours (26/67, 39%, $p = 0.047$), whilst the tumours defined as CIMP+ using two other methods showed no difference in the frequency of global hypomethylation when compared to CIMP-tumours.

3.2. Comparison of clinical and molecular characteristics of MSI and MSS tumours

MSI was present in 10 of 74 tumours (14%). Patients with MSI were older than those with MSS (78.3 ± 9.1 versus. 66.6 ± 13.4, $p = 0.003$, Table 4). Nine of 10 MSI tumours (90%) were located on the proximal side, whilst only 22 of 64 (34%) MSS cancers were on the proximal side ($p = 0.001$) (Table 4). Tumours with MSI also showed significant correlation with *BRAF* mutation, chromosomal stability and inverse correlation with global hypomethylation and survival (Table 4). Of the 10 MSI tumours, six (60%) showed *BRAF* mutations. LOH at either one or more or two or more chromosomal loci was observed in 2 (20%) and 0 (0%) of MSI tumours, respectively. MSI tumours did not show global hypomethylation. Amongst 64 MSS tumours, only four (6%) showed *BRAF* mutations, whilst LOH at either one or more or two or more loci was observed in 51 (80%) and 34 (53%) of tumours, respectively. Global hypomethylation which was absent in MSI tumours was observed in 26 (41%) of MSS tumours. There was no significant correlation between MSI status of tumours and genders, cancer stages, *KRAS* and *p53* mutation status.

Table 4 – Comparison of clinical and molecular characteristics of MSI and MSS tumours

	MSS (%)	MSI (%)	*p*
Number of cases	64	10	
Gender			
M	37 (60)	7 (70)	ns
F	25 (40)	3 (30)	
Age	66.6 ± 13.4	78.3 ± 9.1	0.003
Stage			
A/B	35 (55)	7 (70)	ns
C/D	29 (45)	3 (30)	
Survival (5-year)			
+	36 (56)	2 (20)	0.004
–	28 (44)	8 (80)	
Location			
Proximal	22 (34)	9 (90)	0.001
Distal	42 (66)	1 (10)	
hMLH1 methylation			
+	0 (0)	10 (100)	<0.001
–	64 (100)	0 (0)	
BRAF mutation			
+	4 (6)	6 (60)	<0.001
–	60 (94)	4 (40)	
KRAS mutation			
+	22 (34)	2 (20)	ns
–	42 (66)	8 (80)	
p53 mutation			
+	31 (48)	5 (50)	ns
–	33 (52)	5 (50)	
LOH (⩾1)			
+	51 (80)	2 (20)	<0.001
–	13 (20)	8 (80)	
LOH (⩾2)			
+	34 (53)	0 (0)	0.001
–	30 (47)	10 (100)	
Global hypomethylation			
+	26 (41)	0 (0)	0.012
–	38 (59)	10 (100)	

3.3. Classification of methylated loci based on methylation frequency in tumours with different MSI status and location

Based on methylation frequency in tumours, the 14 loci were classified into three types. In the first type with 7 loci (Type 1), *SFRP1*, *TAC1*, *SST*, *SLC5A8*, *SFRP2*, *SFRP4* and *SFRP5* genes were methylated with similar frequency in both MSI and MSS tumours, and in proximal and distal tumours. The methylation frequency of these genes in tumours were high (80–100%) except for *SFRP4* and *SFRP5*, which showed 51% and 27% methylation frequency, respectively (Table 5). In the second type with 6 loci (Type 2), *RASSF2*, *HIC1*, MINT31, MINT1, *p16ink4A* and *hMLH1* were methylated more frequently in MSI tumours than MSS. These loci have been commonly used in CIMP analysis by many investigators.[9,13] The methylation frequency of these 6 loci was higher in proximal tumours. In MSS tumours, the methylation frequency of these loci was lower than in MSI tumours, and no significant difference in the methylation frequency of these loci was observed between the tumours in proximal and distal colon except for *RASSF2* which was more frequently methylated in proximal colon (Table 5). In the third category (Type 3), the only locus was *MGMT*. It was methylated more frequently in MSI (90%) than in MSS tumours (55%, $p = 0.042$), but methylation occurred more frequently in tumours in distal colon (70%) compared to tumours in proximal colon (45%, $p = 0.054$). In MSS tumours, the methylation frequency of *MGMT* was significantly higher in distal colon compared with proximal colon (69% versus 27%, $p = 0.003$, Table 5).

3.4. Methylation frequency and clinical and molecular characteristics of tumours in distal and proximal colon

The patients with distal colon cancer were younger ($p = 0.034$) and had a better 5-year survival ($p = 0.009$) than patients with proximal cancer (Table 6). Proximal cancers had a higher incidence of CIMP+ as defined by three different methods compared to distal cancers (⩾3/6, 42% versus 12%; ⩾9/14, 42% versus 23%; ⩾12/14, 23% versus 0%). Proximal tumours had a significantly higher frequency of MSI (29% versus 2%, $p = 0.001$), and *BRAF* mutations (29% versus 2%, $p = 0.001$)

Table 5 – Comparison of methylation frequency in CpG island of 14 genes in tumours with different MSI status and location

	Total (%)	MSI (%)	MSS (%)	p	MSI + MSS			MSS		
					Proximal (%)	Distal (%)	p	Proximal (%)	Distal (%)	p
Number of cases	74	10	64		31	43		22	42	
Type 1										
SFRP1	74 (100)	10 (100)	64 (100)	ns	31 (100)	43 (100)	ns	22 (100)	42 (100)	ns
TAC1	72 (97)	10 (100)	62 (97)	ns	31 (100)	41 (95)	ns	22 (100)	40 (95)	ns
SST	67 (91)	9 (90)	58 (91)	ns	27 (87)	40 (93)	ns	19 (86)	39 (93)	ns
SLC5A8	60 (81)	9 (90)	51 (80)	ns	27 (87)	33 (77)	ns	19 (86)	32 (76)	ns
SFRP2	59 (80)	9 (90)	50 (78)	ns	23 (74)	36 (84)	ns	15 (68)	35 (83)	ns
SFRP4	38 (51)	7 (70)	31 (48)	ns	16 (52)	22 (51)	ns	9 (41)	22 (52)	ns
SFRP5	20 (27)	3 (30)	17 (27)	ns	7 (23)	13 (30)	ns	4 (18)	13 (31)	ns
Type 2										
RASSF2	37 (50)	8 (80)	29 (45)	ns	21 (68)	16 (37)	0.018	14 (64)	15 (36)	0.039
HIC1	26 (35)	7 (70)	19 (30)	0.028	15 (48)	11 (26)	0.052	9 (41)	10 (24)	ns
MINT31	23 (31)	8 (80)	15 (23)	0.001	12 (39)	11 (26)	ns	5 (23)	10 (24)	ns
MINT1	19 (26)	9 (90)	10 (16)	<0.001	13 (42)	6 (14)	0.014	5 (23)	5 (12)	ns
p16ink4A	18 (24)	5 (50)	13 (20)	0.056	10 (32)	8 (19)	ns	5 (23)	8 (19)	ns
HMLH1	10 (14)	10 (100)	0 (0)	<0.001	9 (29)	1 (2)	0.001	0 (0)	0 (0)	ns
Type 3										
MGMT	44 (59)	9 (90)	35 (55)	0.042	14 (45)	30 (70)	0.054	6 (27)	29 (69)	0.003

and lower frequencies of LOH (as defined by two different criteria, 55% versus 84%, $p = 0.009$ and 29% versus 58%, $p = 0.018$) and global hypomethylation (19% versus 47%, $p = 0.025$) (Table 6). No significant difference in the frequencies of *KRAS* and *p53* mutations was observed between proximal and distal tumours.

3.5. *Clinical and molecular characteristics of tumours with MGMT methylation*

MGMT gene methylation was observed in 44 of 74 tumours (59%), and was significantly correlated with MSI status of cancers. MSI was present in 9 of 44 *MGMT* methylated tumours (20%), but only in 1 of 30 *MGMT* unmethylated tumours (3%, $p = 0.042$, Table 7). *MGMT* methylation also showed a borderline correlation with a low tumour stage (66% versus 43% for tumours with or without *MGMT* methylation, $p = 0.061$, Table 7). No significant correlation was observed between *MGMT* methylation status of the tumours and the overall mutation frequencies of *KRAS* and *p53*. However, G to A mutations in non-CpG sequences of *KRAS* occurred more frequently in the tumours with *MGMT* methylation than those without *MGMT* methylation (23% versus 10%, $p = 0.2$), and the difference was greater in p53 (32% versus 10%, $p = 0.047$) (Table 8). When the frequency of G to A mutations in non-CpG sequences of both *KRAS* and *p53* was combined together, *MGMT* methylation status of the tumours was significantly correlated with these mutations (55% versus 20% for tumours with or without *MGMT* methylation, $p = 0.004$, Table 8). Although statistically not significant, the tumours with *MGMT* methylation showed a higher incidence of CIMP+ (30% versus 17%), and a lower incidence of global hypomethylation (27% versus 47%) when compared to the tumours without *MGMT* methylation (Table 7). However, *MGMT* methylation of the tumours did not show correlation with gender, age at diagnosis, *BRAF* gene mutations or LOH (Table 7).

4. Discussion

When the frequency of CpG island methylation was examined in colorectal cancers using 14 loci consisting of six commonly used loci and eight recently identified tumour associated methylation loci, methylation was found to be a frequent event, but considerable variation in methylation frequency amongst tumours and individual loci was observed. The tumours were classified into three groups, low, high and very high methylation groups based on methylation frequency. The tumours with very high methylation more frequently showed a proximal location, MSI, *BRAF* mutations and less frequent LOH and global hypomethylation, compared with tumours with low methylation, whereas tumours with high methylation showed intermediate levels of these characteristics. These results are consistent with previous reports and add further support to the existence of significant positive association of frequent promoter methylation with MSI, *BRAF* mutation and proximal location and of negative association with LOH in colorectal cancers.[4,8,9,13]

A subset of colorectal cancers has been observed to have a high frequency of cancer specific methylation of some CpG islands of multiple genes (type C) and referred to as CIMP.[9,13] However, the concept of CIMP has been challenged as being related to ageing rather than a neoplastic process or to *MLH1* methylation or MSI.[14,15] In order to gain further insight into CIMP, we arbitrarily classified CIMP according to methylation frequency of 6 traditional type C loci (three or more loci), or that of 14 loci including six 'type C' loci and eight recently characterised additional colorectal cancer-associated loci (9 or more or 12 or more loci). All tumours with CIMP defined

Table 6 – Comparison of clinical and molecular characteristics of proximal and distal tumours

	Proximal (%)	Distal (%)	*p*
Number of cases	31	43	
Gender			
M	20 (65)	24 (59)	ns
F	11 (35)	17 (41)	
Age	72.2±13.7	65.3±12.6	0.034
Stage			
A/B	16 (52)	26 (60)	ns
C/D	15 (48)	17 (40)	
Survival (5-year)			
+	10 (32)	28 (65)	0.009
–	21 (68)	15 (35)	
CIMP (⩾9/14)			
+	13 (42)	10 (23)	ns
–	18 (58)	33 (77)	
CIMP (⩾12/14)			
+	7 (23)	0 (0)	0.001
–	24 (77)	43 (100)	
MSI			
+	9 (29)	1 (2)	0.001
–	22 (71)	42 (98)	
BRAFk mutation			
+	9 (29)	1 (2)	0.001
–	22 (71)	42 (98)	
KRAS mutation			
+	9 (29)	15 (35)	ns
–	22 (71)	28 (65)	
p53 mutation			
+	15 (48)	21 (49)	ns
–	16 (52)	22 (51)	
LOH (⩾1)			
+	17 (55)	36 (84)	0.009
–	14 (45)	7 (16)	
LOH (⩾2)			
+	9 (29)	25 (58)	0.018
–	22 (71)	18 (42)	
Global hypomethylation			
+	6 (19)	20 (47)	0.025
–	25 (81)	23 (53)	

by three different criteria showed positive association with MSI, *BRAF* mutations and negative association with LOH. Interestingly, global hypomethylation was negatively associated with CIMP only when it was defined as 12 or more of 14 loci being methylated. This indicates that inverse relationship between CIMP and global hypomethylation exists only when CIMP is defined by a very high degree of methylated loci in the tumour. All tumours with CIMP also tended to show the proximal location more frequently. Thus, our results indicate that a subset of tumours with CIMP having distinct molecular and clinical characteristics indeed appears to exist. The frequency of colorectal cancers with CIMP ranged from 10% to 31% in our study depending on the criteria that we used in defining CIMP, such as the type and number of methylated loci.

Table 7 – Clinical and molecular characteristics of tumours with and without MGMT methylation

	MGMT met – (%)	MGMT met + (%)	*p*
Number of cases	30	44	
Gender			
M	19 (63)	25 (60)	ns
F	11 (37)	17 (40)	
Age	67.5±14.4	68.9±12.9	ns
Stage			
A/B	13 (43)	29 (66)	0.061
C/D	17 (57)	15 (34)	
Survival (all patients)			
+	11 (37)	27 (61)	0.058
–	19 (63)	17 (39)	
Survival (MSS patients)			
+	10 (34)	26 (74)	0.002
–	19 (66)	9 (26)	
Location			
Proximal	17 (57)	14 (32)	0.054
Distal	13 (43)	30 (68)	
MSI			
+	1 (3)	9 (20)	0.042
–	29 (97)	35 (80)	
CIMP (⩾3/6)			
+	5 (17)	13 (30)	ns
–	25 (83)	31 (70)	
BRAF mutation			
+	4 (13)	6 (14)	ns
–	26 (87)	38 (86)	
KRAS mutation (all)			
+	9 (30)	15 (34)	ns
–	21 (70)	29 (66)	
p53 mutation (all)			
+	12 (40)	24 (55)	ns
–	18 (60)	20 (45)	
LOH (⩾1)			
+	22 (73)	31 (70)	ns
–	8 (27)	13 (30)	
LOH (⩾2)			
+	16 (53)	18 (41)	ns
–	14 (47)	26 (59)	
Global hypomethylation			
+	14 (47)	12 (27)	ns
–	16 (53)	32 (73)	

Based on the frequency of methylation, MSI and tumour location, we were also able to classify 14 loci into three types. Type 1 loci tended to get methylated at higher frequency than Type 2 and Type 3 regardless of MSI status and tumour location, whilst Type 2 loci showed a higher frequency of methylation in tumours with MSI or proximal location. Methylation of Type 3 locus, *MGMT* occurred more frequently in tumours with MSI or distal location. These results suggest locus specific phenotype changes of tumours and underscore the existence of considerable variation amongst different loci in the frequency of methylation and the association of locus specific

Table 8 – Correlation of MGMT methylation with KRAS and p53 mutations

	Total (%)	MGMT met – (%)	MGMT met + (%)	p
Number of cases	74	30	44	
KRAS mutation				
All*				
+	24 (32)	9 (30)	15 (34)	ns
–	50 (68)	21 (70)	29 (66)	
Non G to A (at non-CpG site)				
+	11 (15)	6 (20)	5 (11)	ns
–	63 (85)	24 (80)	39 (89)	
G to A (at non-CpG site)				
+	13 (18)	3 (10)	10 (23)	ns
–	61 (82)	27 (90)	34 (77)	
p53 mutation				
All				
+	36 (49)	12 (40)	24 (55)	ns
–	38 (51)	18 (60)	20 (45)	
Non G to A				
+	6 (8)	2 (7)	4 (9)	ns
–	68 (92)	28 (93)	40 (91)	
G to A				
+	30 (41)	10 (33)	20 (45)	ns
–	44 (59)	20 (67)	24 (55)	
At CpG site				
+	13 (18)	7 (23)	6 (14)	ns
–	61 (82)	23 (77)	38 (86)	
At non-CpG site				
+	17 (23)	3 (10)	14 (32)	0.047
–	57 (77)	27 (90)	30 (68)	
KRAS/p53 mutation				
G to A at non-CpG site				
+	30 (41)	6 (20)	24 (55)	0.004
–	44 (59)	24 (80)	20 (45)	

* All mutations are located at non-CpG sites.

methylation of tumours with distinct clinical and molecular characteristics.

In the present study, proximal tumours showed significantly older age at diagnosis, a lower 5-year survival rate, higher frequency of *BRAF* mutation and a higher frequency of methylation at Type 2 loci, and lower frequency of *MGMT* methylation and LOH compared with distal tumours. Since 9 of 10 (90%) of tumours with MSI were located in the proximal location compared to more frequent distal locations of 42 of 64 (66%) MSS tumours, prevalence of MSI in proximal colon probably influenced our results. When only MSS tumours were considered, no regional difference was observed in age at diagnosis or in the frequency of methylation at Type 2 loci or LOH. However, MSS tumours in the proximal colon still showed a poorer 5-year survival rate, a lower frequency of *MGMT* methylation and a higher incidence of *BRAF* mutation compared with distal MSS tumours. These results provide further support theory that the location of the tumours may be an important determinant of clinical and molecular characteristics of the tumours.[28–31]

MGMT is a DNA repair gene, which is frequently inactivated by promoter methylation in colorectal cancers.[24] Our study showed that *MGMT* methylation occurs frequently (59%) in colorectal tumours and that it has locus specific clinical and molecular features, and was classified as Type 3 methylated locus. *MGMT* methylation was associated with a better 5-year survival rate and distal location, and this association became highly significant when only MSS tumours with *MGMT* methylation were considered. A positive correlation between *MGMT* methylation and MSI was also observed. In the absence of *MGMT* activity resulting from promoter methylation O^6-methylguanine misrepairs with thymine during DNA replication, resulting in G to A mutations. There have been conflicting reports regarding the relationships between *MGMT* methylation and G to A mutations in *KRAS* and *p53* genes.[32–35] We observed no significant correlation between *MGMT* methylation and the frequency of G to A mutations in *KRAS* or *p53* genes. G to A mutations occur only in the non-CpG sites in *KRAS*, but in *p53*, these mutations occur in both CpG and non-CpG sites. Our study showed a strong correlation between *MGMT* methylation and G to A mutations only in the non-CpG sites of *p53*. An even stronger correlation was observed between *MGMT* methylation and G to A mutations in non-CpG sites of both *KRAS* and *p53*. G to A mutations in non-CpG sites of *KRAS* and *p53* did not occur in the same tumour. These results suggest that *MGMT* protects against mutagenic effects of O^6-methylguanine mainly in non-CpG sites, whilst the protection in CpG sites may require other mechanisms.

The observations that CIMP+ correlated with *BRAF* mutations, MSI, proximal location and chromosomal stability (LOH–) were reported in two recent studies.[36,37] Our study supports these observations. However, these studies did not focus on the presence of methylation (at some loci) in cases classified as CIMP negative or CIMP low. In our study, we used an expanded panel of 14 loci and showed that methylation was detected in at least three markers per tumour in all tumours, indicating that cancer-associated methylation is present in all colorectal cancers including CIMP negative or CIMP low tumours as defined by traditional markers. Our observations indicate that methylation at some loci occurs in all colorectal cancers regardless of CIMP status. In our previous study, we identified the distinct genetic and epigenetic differences between mucinous and non-mucinous colorectal cancer by comparing the frequency of MSI, CIMP (using traditional markers) and *BRAF* mutations.[38] In the present study, we determined methylation status in a wider panel of markers (six traditional markers and eight additional genes), as well as *p53*, *KRAS* and *BRAF* gene mutations by sequencing, and analysed LOH and global hypomethylation status. We then compared these data with clinical and other molecular characteristics of tumours, and found that the methylation profiles were inversely correlated with LOH and global hypomethylation. We further identified distinct gene-specific methylation profiles and clinical and molecular characteristics between proximal and distal colorectal cancer. In addition, we observed that *MGMT* methylation was correlated with G to A mutations in *KRAS* and *p53* gene, and methylation of a group of genes (Type 1) was commonly present in all colorectal cancers regardless of CIMP status.

The simultaneous methylation of *p16* and *MGMT* genes was reported in 23% of colorectal cancers[39], and was associ-

ated with lower occurrence of metastasis and/or death within 2-year period. In our study, we observed that methylation of *MGMT* gene was significantly correlated with 5-year survival in MSS patients, and co-methylation of *p16* and *MGMT* genes was present in 10 of 74 (14%) tumours. However, co-methylation of *p16* and *MGMT* genes was not correlated with 5-year survival. These different observations may in part be due to different geographic origin of the patients, or to different time period used for determining the patient's survival.

In summary, methylation analysis of a panel of 14 genes indicates that methylation is a frequent event in colorectal carcinogenesis and that gene-specific methylation patterns of the tumours are associated with characteristic clinical and molecular features. Moreover, our data show that proximal or distal locations of colorectal cancers may be an important determinant of gene-specific methylation profiles and the molecular and clinical characteristics of these tumours.

Conflict of interest statement

None declared.

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