Table 1. Primer sequences for methylation-specific PCR and for pyrosequencing.

Primer sequence	Anneal (°C)	Product (bp)	Number of analyzed CpG sites	Primer position
ADAMTS1 (Bottom strand)				
Fwd: GTTTCGAGATTTCGGAGTTCGTTTCGC	64	97	5	+538 to +512
Rev[1]: AAACTCCAATACAACGAACTATACCCG			2	+470 to +442
Seq: TTTTTTATGTAGTTGTTTAGTT			2	+510 to +499
STOX2 (Top strand)				
Fwd[1]: TGGGGTAGTTGTTAAGGTTTTCGCGTC	61	97	3	+301 to +327
Rev: CACCAAACTACCTTAAATTAAAACGCG			2	+371 to +397
Seq: CATCAAACTTCTCATTTTCATATA			4	+375 to +352
EDIL3 (Bottom strand)				
Fwd: GATTAAGAGTTAGACGGTTATCGAGC	64	79	3	+452 to +427
Rev[1]: CGCGACGACCCCTAACCAACCGAAATCACG			5	+403 to +374
Seq: GGTTATAGAGAGTTTTATGATTT			2	+437 to +415
COL4A2 (Bottom strand)				
Fwd: TTTATCCTCGGTTTCGGTTC	64	72	3	+529 to +510
Rev[1]: CTCCCATCACCCCTACATACG			1	+478 to +458
Seq: GAGAAGAGGGGATAG			4	+507 to +493
PPP1R3C (Top strand)				
Fwd: TCGTTTCGGGGCGATTACGTTGTC	65	100	5	−123 to −120
Rev[1]: CCTAAAACCAATCGCCGAACCTCG			3	−47 to −24
Seq: GAGGGTTGGAGTTTTAGTTGG			3	−114 to −94
EFHD1 (Top strand)				
Fwd: TTTCGAGTTTGCGAGGAGCGCGTC	68	90	5	+4 to +27
Rev[1]: CATAACGACGAATCGCAAAACGCG			5	+70 to +93
Seq: CGTCGTTAGTTAGTTTTTTG			6	+24 to −43
TSPY5 (Top strand)				
Fwd: TATAGTTGTACGTTCGTGACGTC	61	75	4	−17 to +6
Rev[1]: CCTAACGCCAACTCTCGATCG			3	+38 to +58
Seq: GGTTGTAGTGGAGAGATT			4	+10 to +27

The position of the transcription start site (TSS) was regarded as +1. The DNA strand used for the template was shown by *top/bottom*. *Fwd/Rev*, forward and reverse primers for methylation-specific PCR; *Seq*, sequence primer for pyrosequencing; *C/G*, C in forward primer and G in reverse primer to distinguish methylated DNA from unmethylated DNA.
[1]Primers biotinylated for pyrosequencing.

product was obtained in the 0% control sample and that the fully methylated allele was amplified in the 100% control sample.

Evaluation of protein markers CEA and CA19-9

At clinical diagnosis of CRC, serum CEA and CA19-9 levels were evaluated by Enzyme-linked immunosorbent assay. CEA and CA19-9 were considered positive when CEA was ≥5 ng/mL and CA19-9 was ≥40 U/mL.

Statistical analysis

P-values were calculated to compare CRC patients and noncancer patients. Student's *t*-test was used for age and Fisher's exact test was used for analysis of sex. *P*-values were also calculated to compare methylation(+) group and methylation(−) group. Student's *t*-test was used for age and Fisher's exact test was used for analysis of sex, AJCC stage, neoadjuvant chemotherapy, and tumor locations (Tables 2 and 3). In each AJCC stage, methylation frequency in plasma DNA samples was also compared with frequencies of CEA(+) and CA19-9(+) using Fisher's exact test (Fig. 6). When $P < 0.05$, the correlation was considered statistically significant. Student's *t*-test and Fisher's exact test were performed using R software (www.r-project.org/).

Results

Selection of candidate genes

In our previous methylome analysis of CRC, 60 methylation genes to epigenotype CRC were established and their methylation levels were analyzed quantitatively in 149 CRC and nine normal colon samples [15]. Among them, 12 genes were not hypermethylated in any of the normal

Table 2. *PPP1R3C* methylation and clinicopathological factors.

	Methylated	Unmethylated	*P*-value
Number	97	23	
Age (years)	67.9 ± 11.4	67.0 ± 11.8	0.9
Sex (male/female)	59/38	12/11	0.7
AJCC stage			0.7
I/II/III/IV	11/23/9/54	1/7/3/12	
NAC (yes/no)	17/80	3/20	0.5
Tumor location			0.4
Proximal (Ce/A/T)	36 (9/11/16)	5 (1/4/0)	
Distal (D/S)	29 (3/26)	8 (1/7)	
Rectum	32	10	

Age was shown by mean ± standard deviation. AJCC, American Joint Committee on Cancer; NAC, neoadjuvant chemotherapy. Tumor locations were classified into proximal colon including cecum (Ce), ascending (A) and transverse colon (T), distal colon including descending (D) and sigmoid colon (S), and rectum. *P*-values were analyzed using the Student's *t*-test for age and the Fisher's exact test for sex, stage, NAC, and tumor location.

Table 3. *EFHD1* methylation and clinicopathological factors.

	Methylated	Unmethylated	*P*-value
Number	75	45	
Age (years)	67.0 ± 10.7	71.2 ± 12.3	0.5
Sex (male/female)	44/31	27/18	0.4
AJCC stage			1.0
I/II/III/IV	7/18/8/42	5/12/4/24	
NAC (yes/no)	13/62	7/38	0.7
Tumor location			0.4
Proximal (Ce/A/T)	26 (7/9/10)	15 (3/6/6)	
Distal (D/S)	20 (2/18)	17 (2/15)	
Rectum	29	13	

Age was shown by mean ± standard deviation. AJCC, American Joint Committee on Cancer; NAC, neoadjuvant chemotherapy. Tumor locations were classified into proximal colon including cecum (Ce), ascending (A) and transverse colon (T), distal colon including descending (D) and sigmoid colon (S), and rectum. *P*-values were analyzed using the Student's *t*-test for age and the Fisher's exact test for sex, stage, NAC, and tumor location.

colon samples, but were frequently methylated (>75%) in CRC cases: *COL4A2* (147/149), *TSPYL5* (141/149), *TMEFF2* (141/149), *RASSF2* (134/149), *SPG20* (130/149), *EDIL3* (130/149), *CIDEB* (128/149), *ADAMTS1* (128/149), *EFHD1* (127/149), *STOX2* (126/149), *PPP1R3C* (118/149), and *UCHL1* (115/149) (Fig. 1). *CDO1*, *SFRP1*, and *PENK1* showed frequent hypermethylation in >75% of CRC cases, but were also aberrantly methylated in normal samples. Although the size of the normal samples was as small as nine, the former 12 genes were extracted as candidate genes because of no hypermethylation in normal samples, and the latter three genes were excluded.

To detect aberrantly methylated alleles, bisulfite-treated genomic DNA was amplified using methylation-specific PCR primers designed to generate PCR products ≤100 bp. To validate that methylation-specific PCR products resulted from amplification of methylated alleles, and not from unexpected amplification of unmethylated DNA or DNA with partial methylation in primer regions, sequence primers were designed within the product regions and the methylation level of the PCR products were analyzed using pyrosequencing. Such primers for methylation-specific PCR and pyrosequencing were successfully designed for seven of the 12 genes: *COL4A2*, *TSPYL5*, *EDIL3*, *ADAMTS1*, *EFHD1*, *STOX2*, and *PPP1R3C* (Table 1).

In pyrosequencing, the signal intensity should be high enough (≥5) and detected methylation rate should be high enough (60–100%) if methylated allele was successfully amplified. If methylation rate were low (<60%), that would be due to unexpected amplification of unmethylated allele in methylation-specific PCR, and the sample would therefore be regarded as methylation(−). But all the analyzed samples showed methylation rate as high as 60–100% when the signal intensity was higher than 5, and they were regarded as methylation(+). When no signal was detected in pyrosequence, that should be due to no amplification in methylation-specific PCR, the sample was regarded as methylation(−). When the signal intensity was too low to accurately calculate methylation rate, that would be regarded as insufficient amplification by methylation specific PCR, we set the threshold of the signal intensity at 5; the sample was regarded as methylation (−) when the signal intensity was <5 (Fig. S1). To check the quality of sample DNA, bisulfite-converted DNA was amplified using primers for *MYOD* upstream region. *MYOD* primers were designed in the regions without CpG sites, and therefore amplify the region regardless methylation status. All the analyzed samples showed amplification of the *MYOD* region, indicating that lack of amplification is due to absence of methylation, not due to poor DNA quality (Fig. S1).

Selection of genes using normal peripheral blood cell samples

Considering that plasma DNA samples can be easily contaminated with DNA originating from normal peripheral blood cells, we first analyzed the methylation status of the seven genes in peripheral blood cell samples from four noncancer patients. Methylation of *PPP1R3C* and *EFHD1* was rarely detected in peripheral blood cells, but the other five genes, *STOX2*, *EDIL3*, *COL4A2*, *TSPYL5*, and *ADAMTS1*, were frequently methylated in these cells (Fig. 2). Given that false-positive results could potentially be

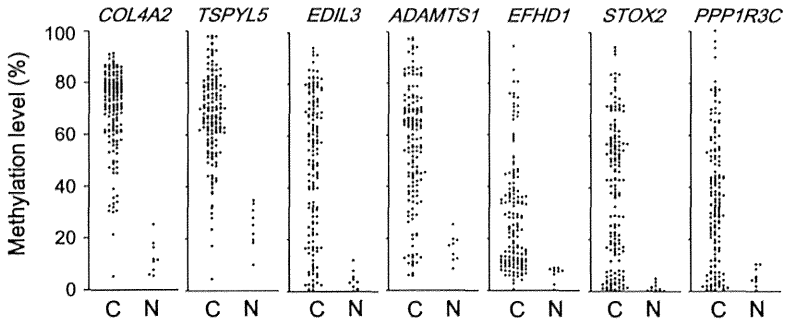


Figure 1. Candidate genes frequently hypermethylated in CRC. Using quantitative methylation data from 149 CRC and nine normal colon samples conducted in our previous analysis [15], we selected 12 genes that were not hypermethylated in normal colon (methylation level <35% in all nine samples), but aberrantly methylated in most of the 149 CRC cases (methylation level >35% in 112 or more samples). Seven genes, for which primers could be designed, are representatively shown.

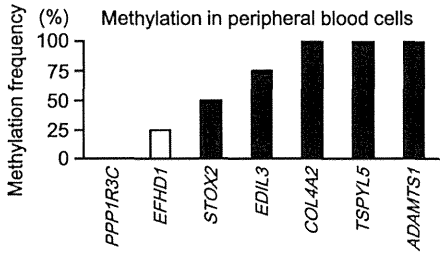


Figure 2. Screening of seven genes using peripheral blood cells. Methylation status was analyzed using peripheral blood cell samples from four noncancer patients. *PPP1R3C* and *EFHD1* showed no or infrequent methylation (*open box*), but the other five genes showed methylation frequency in peripheral blood cells (*closed box*).

obtained if these latter five genes were analyzed in plasma DNA samples, *PPP1R3C* and *EFHD1* were selected for subsequent analyses.

Methylation of *PPP1R3C* and *EFHD1* in plasma and tumor samples from CRC patients

PPP1R3C and *EFHD1* were analyzed using plasma samples from 24 CRC patients and their corresponding CRC tissue samples (Fig. 3). *PPP1R3C* and *EFHD1* were methylated in 22 (92%) and 19 (79%) of the 24 CRC tissue samples, respectively. While these two genes were frequently methylated in 149 CRC tissue samples in the previous study, it was confirmed that they were also frequently methylated in this additional set of CRC tissue samples. When plasma DNA samples from these CRC patients were analyzed, *PPP1R3C* and *EFHD1* were frequently methylation-positive (+), at 79% (19/24) for each gene (Fig. 3A).

When the two genes were combined, all 24 (100%) CRC tissue samples and 22 (94%) plasma DNA samples were methylation(+) for at least one of the two genes

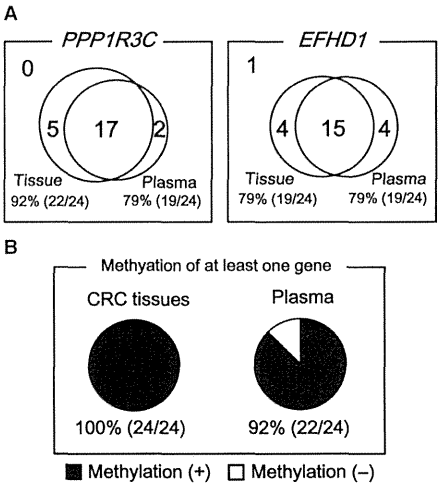


Figure 3. Screening of two genes using CRC tissue and corresponding plasma samples. (A) Methylation status in plasma and CRC tissue samples from 24 CRC patients. In another set of 24 CRC tissue samples than those in Figure 1, *PPP1R3C* and *EFHD1* were confirmed to be frequently methylated at 92% (22/24) and 79% (19/24), respectively. The corresponding plasma samples were also frequently methylated at 79% (19/24) for each gene. (B) Frequency of the methylation of at least one gene. Among 24 patients, at least one of the two genes was methylated in 24 (100%) CRC tissue and 22 (92%) plasma samples.

(Fig. 3B). This suggested that high sensitivity could be obtained if these two genes were analyzed for CRC detection.

A small number of cases were methylation(+) in plasma DNA samples despite methylation(−) in CRC tissue samples. These might be due to unexpected methylation in peripheral blood cells contaminated in plasma samples or it might be due to heterogeneity of tumor tissues, that is, plasma DNA derived from a part of CRC

might be methylated while the analyzed piece of CRC tissue might not be methylated.

Comparison between CRC patients and noncancer patients

Next, *PPP1R3C* and *EFHD1* were analyzed using plasma samples from 120 CRC patients and 96 noncancer patients. *PPP1R3C* was methylated in 81% (97/120) of CRC patients (Fig. 4), which was at a similar frequency determined for the initial 24 samples (Fig. 3). The methylation(+) ratio for noncancer patients was 19% (18/96) ($P = 6 \times 10^{-20}$, Fisher's exact test). *EFHD1* was methylated in 62% (75/120) of CRC patients and in 22% (21/96) of noncancer patients ($P = 3 \times 10^{-9}$) (Fig. 4).

If analyses of these two genes were combined, then at least one gene was methylated in 90% (108/120) of CRC patients and in 36% (35/96) of noncancer patients ($P = 4 \times 10^{-17}$). Both *PPP1R3C* and *EFHD1* genes were methylated in 53% (64/120) of CRC patients, but in only 4% (4/96) of noncancer patients ($P = 2 \times 10^{-16}$) (Fig. 5A).

When a single gene was used for CRC detection using plasma samples, *PPP1R3C* gave better results than *EFHD1*. For *PPP1R3C*, 97 (81% sensitivity) of 120 CRC patients and 78 (81% specificity) of 96 noncancer patients were diagnosed correctly. The sensitivity and specificity could be improved when *EFHD1* was combined with *PPP1R3C*. If methylation of at least one gene was regarded as methylation(+), as many as 108 of 120 CRC patients would have been diagnosed correctly, with 90% sensitivity. If methylation of both genes was regarded as methylation(+), as many as 92 of 96 noncancer patients would have been diagnosed correctly, with 96% specificity, while the sensitivity would be 53% (Fig. 5B).

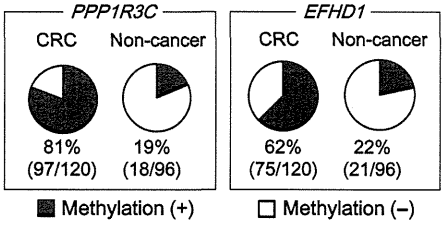
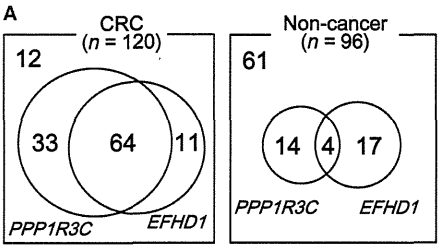


Figure 4. Methylation in plasma samples from 122 CRC patients and 96 noncancer patients. *PPP1R3C* was methylated in 81% (97/120) of CRC patients and in 19% (18/96) of noncancer patients ($P = 6 \times 10^{-20}$, Fisher's exact test). *EFHD1* was methylated in 62% (75/120) of CRC patients and in 22% (21/96) of noncancer patients ($P = 3 \times 10^{-9}$).



B

	PPP1R3C	*EFHD1*	*PPP1R3C* or *EFHD1*	*PPP1R3C* and *EFHD1*
Sensitivity	81%	63%	90%	53%
Specificity	81%	78%	64%	96%

Figure 5. Sensitivity and specificity of the two methylation genes. (A) Combination of the two genes. Frequency of methylation of both genes was 53% (64/120) for CRC patients, but only 4% (4/96) for noncancer patients ($P = 2 \times 10^{-16}$, Fisher's exact test), giving high specificity. Frequency of methylation of at least one of the two genes was 90% (108/120) for CRC patients, but only 36% (35/96) for noncancer patients ($P = 4 \times 10^{-17}$), giving high sensitivity. (B) Sensitivity and specificity. Methylation of *PPP1R3C* gave better sensitivity and specificity, 81% and 81%, respectively, than did *EFHD1*. When the frequency of methylation of at least one gene was analyzed, sensitivity was increased to 90%. When the frequency of methylation of both genes was analyzed, specificity was as high as 96%.

Comparison with protein markers, CEA and CA19-9

To evaluate the usefulness of the two methylation genes, their sensitivities were compared with two protein markers, CEA and CA19-9 (Fig. 6). CEA and CA19-9 were positive in 64% (77/120) and 34% (41/120) of CRC cases, respectively. *PPP1R3C* methylation showed a higher sensitivity, 81% (97/120), than the two protein markers. At early clinical stages, sensitivity of *PPP1R3C* methylation was significantly higher than the protein markers (Fig. 6A). For stage I CRC, 92% (11/12) samples were *PPP1R3C* methylation(+), whereas only 17% (2/12) were CEA(+) ($P = 3 \times 10^{-4}$, Fisher's exact test) and 0% (0/12) was CA19-9(+) ($P = 5 \times 10^{-6}$). For stage II CRC, 77% (23/30) were *PPP1R3C* methylation(+), whereas only 40% (12/30) were CEA(+) ($P = 0.004$) and 13% (4/30) were CA19-9(+) ($P = 7 \times 10^{-7}$).

Sensitivity of *EFHD1* methylation was also significantly higher than the protein markers for stage I CRC. Seven (58%) of 12 were *EFHD1* methylation(+), whereas 17% were CEA(+) ($P = 3 \times 10^{-4}$) and 0% was CA19-9(+) ($P = 5 \times 10^{-6}$) (Fig. 6B).

When *EFHD1* methylation was combined with *PPP1R3C* analysis and methylation of at least one gene was regarded as methylation(+), the sensitivity at early

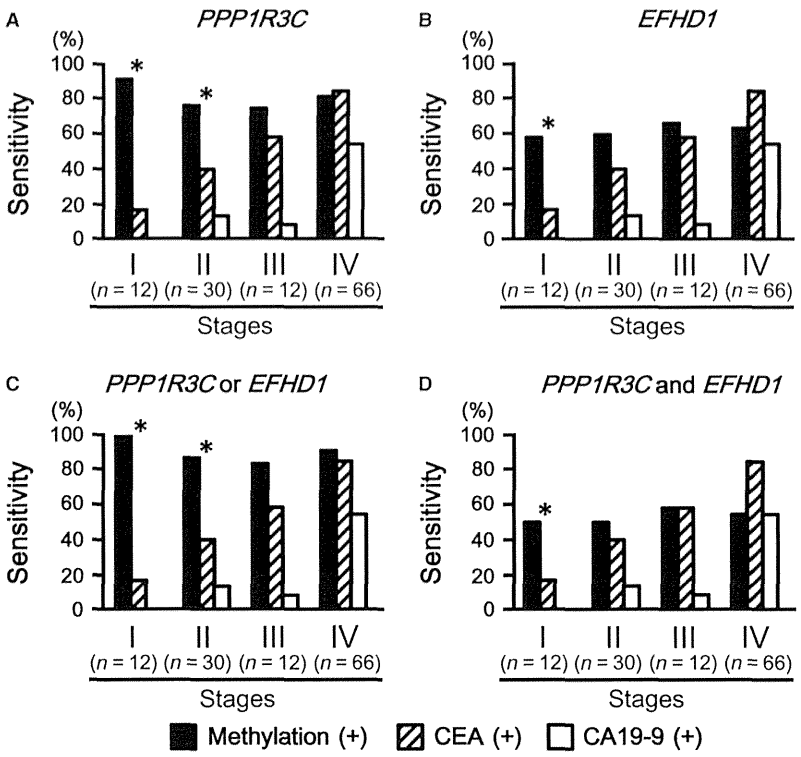


Figure 6. Comparison of the methylation genes with tumor markers, CEA and CA19-9. *Closed box*, methylation; *hatched box*, CEA; *open box*, CA19-9. While sensitivities for CEA and CA19-9 were 64% (77/120) and 34% (41/120), respectively, methylation showed a higher sensitivity, especially at early clinical stages. *$P < 0.05$, between methylation and CEA and between methylation and CA19-9. (A) Methylation of *PPP1R3C*. For stage I, 11 (92%) of 12 CRCs were *PPP1R3C* methylation(+), whereas 2 (17%) of 12 CRCs were CEA(+) ($P = 3 \times 10^{-4}$, Fisher's exact test), and 0% (0/12) were CA19-9(+) ($P = 5 \times 10^{-6}$). For stage II, 23 (77%) of 30 CRCs were *PPP1R3C* methylation(+), whereas 40% (12/30) were CEA(+) ($P = 0.004$) and 13% (4/30) were CA19-9(+) ($P = 7 \times 10^{-7}$). (B) Methylation of *EFHD1*. For stage I, 7 (58%) of 12 CRCs were *EFHD1* methylation(+) ($P = 3 \times 10^{-4}$ against CEA, $P = 5 \times 10^{-6}$ against CA19-9). (C) Methylation of at least one gene. For stage I, 12 (100%) CRCs were methylation(+) ($P = 3 \times 10^{-5}$ against CEA, $P = 4 \times 10^{-7}$ against CA19-9). For stage II, as many as 26 (87%) of 30 CRCs were methylation (+) ($P = 2 \times 10^{-4}$ against CEA, $P = 6 \times 10^{-9}$ against CA19-9). (D) Methylation of both *PPP1R3C* and *EFHD1*. For stage I, 6 (50%) CRCs were methylation(+) ($P = 3 \times 10^{-5}$ against CEA, $P = 4 \times 10^{-7}$ against CA19-9).

clinical stages was further increased. All 12 (100%) were methylation(+) for stage I CRC ($P = 3 \times 10^{-5}$ against CEA and $P = 4 \times 10^{-7}$ against CA19-9). For stage II CRC, 87% (26/30) were methylation(+) ($P = 2 \times 10^{-4}$ against CEA and $P = 6 \times 10^{-9}$ against CA19-9) (Fig. 6C). Even when methylation of both *PPP1R3C* and *EFHD1* was regarded as methylation(+), resulting in very high specificity, the sensitivity for stage I CRC was still significantly higher than that of the protein markers. Six (50%) of 12 CRCs were methylation(+), whereas 17% were CEA(+) ($P = 3 \times 10^{-5}$) and 0% was CA19-9(+) ($P = 4 \times 10^{-7}$).

Comparison with other clinicopathological factors

Methylation status of *PPP1R3C* and *EFHD1* was compared with other clinicopathological factors including sex, age, tumor stage, and tumor locations (Tables 2 and 3). For both genes, methylation(+) and methylation(−) cases did not show significant difference in sex, age, tumor stage, presence or absence of neoadjuvant chemotherapy, and tumor locations.

Discussion

Aberrant DNA methylation of promoter CpG islands is one of major epigenetic alterations in CRC [9, 10]. Some genes are commonly methylated in CRC regardless of epigenotypes and could possibly be utilized as CRC detection markers. Among these commonly methylated genes, ones methylated in normal colon samples or in peripheral blood cells were excluded. *PPP1R3C* and *EFHD1* were selected and subsequently analyzed using plasma DNA samples of 120 CRC and 96 noncancer patients, using methylation-specific PCR in combination

with pyrosequencing for validation of specific amplification of methylated DNA. Detection of *PPP1R3C* methylation alone or its combination with *EFHD1* methylation in plasma DNA samples was found to show high sensitivity and specificity, and their sensitivities in early-stage CRCs were substantially higher than that of CEA and CA19-9.

In 2004, Müller et al. assessed *SFRP2* methylation in fecal DNA to diagnose CRC using MethyLight analysis; its sensitivity and specificity were as high as 77% and 77%, respectively, although they analyzed only 13 CRC and 13 control samples [5]. In 2005, Chen et al. analyzed *VIM* methylation in fecal DNA from 94 CRC and 198 control samples using methylation-specific PCR; its specificity was as high as 90%, while sensitivity was 46% [24]. As for methylation in plasma DNA, Lofton-Day et al. searched for CRC-specific methylated DNA in plasma and reported that the sensitivity and specificity of *TMEFF2*, *NGFR*, and *SEPT9* were 65% and 69%, 51% and 84%, and 69% and 86%, respectively [11]. When *PPP1R3C* methylation was used alone in this study, its sensitivity (81%) and specificity (81%) were considerably high, compared with these reports.

Several groups analyzed *SEPT9* methylation in plasma samples for CRC detection. Some reports showed considerably high sensitivity (90–96%) and specificity (85–88%) [12, 25], while other groups reported relatively lower sensitivity (48–72%) but higher specificity (86–95%) [26–28]. In 2009, deVos et al. measured SEPT9 methylation using real-time PCR-based analysis, in which three independent experiments per sample were performed. High-sensitivity method, where at least one of three PCR was positive, resulted in 72% sensitivity and 86% specificity. But high-specificity method, where at least two of three PCRs were positive, resulted in 56% sensitivity and 95% specificity [27]. This indicated that the results were dependent on the decision criteria, and that specificity would be increased by lowering sensitivity. Our results had similar tendencies. In high-sensitivity analysis where methylation of at least one gene was regarded as methylation(+), sensitivity improved to 90% while specificity was 64%. In high-specificity analysis where methylation of both the *PPP1R3C* and *EFHD1* genes was regarded as methylation(+), specificity improved to as high as 96% while sensitivity was 53%. These suggested that in addition to *SEPT9* methylation, *PPP1R3C* methylation alone or in combination with *EFHD1* methylation could be detection markers for CRC detection with high sensitivity and high specificity.

CRC is one of the leading causes of cancer deaths in the world, and diagnosis at an early onset followed by surgical intervention is currently the best way to cure the disease and decrease mortality. It is therefore important to develop detection markers to detect asymptomatic CRCs at earlier stages, while the sensitivities of CEA and CA19-9 were reported to be relatively low in early-stage CRCs [3]. Our previous studies of DNA methylation in CRC and precancerous lesions revealed that accumulation of aberrant DNA methylation was mostly completed by the adenoma stage [15, 29], suggesting the possible usefulness of assessing aberrant methylation in plasma DNA in detecting early-stage CRCs. Warren et al. reported that the sensitivity of *SEPT9* methylation was 71% for stage I CRCs [12]. In another report, the sensitivity of *SEPT9* methylation was 60% for stage I CRCs, which could be increased to 84% using a high-sensitivity method [25]. *PPP1R3C* methylation in this study gave a similar or even better results in detecting early-stage CRCs. The sensitivity of methylation of *PPP1R3C* alone was 92% for stage I CRCs. Using a more sensitive method to detect methylation of at least one of the *PPP1R3C* and *EFHD1* genes, the sensitivity increased to 100% for stage I CRCs. Even in a method with high specificity of 96%, the sensitivity of methylation of both genes was 50% for stage I CRCs, which was significantly higher than sensitivities of CEA (17%) and CA19-9 (0%). This indicated that detection of aberrant methylation in plasma DNA was a powerful method to diagnose CRC, especially for early-stage CRCs, and that *PPP1R3C* and *EFHD1* were useful biomarkers for the method.

A subgroup of methylation genes including CIMP markers were specifically hypermethylated in CIMP(+) high-methylation CRC, and methylation of these genes significantly associated with female, older age, and proximal tumor location [15]. But the genes analyzed in this study were extracted from genes hypermethylated commonly in CRC regardless of epigenotypes, and methylation of these genes did not show significant correlation with sex, age, or tumor location [15]. In good agreement with these previous observation, methylation of *PPP1R3C* and *EFHD1* in plasma DNA samples were detected commonly in CRC patients, regardless of sex, age, or tumor location (Tables 2 and 3).

In summary, detection of methylation of *PPP1R3C* alone or in combination with *EFHD1* in plasma DNA showed high sensitivity and specificity in CRC detection, and may be useful detection method for CRC, especially for early-stage CRCs.

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Conflict of Interest

Ayako Sakai is employed by Sysmex Corporation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative results of pyrosequencing analysis for methylation-specific PCR products of *PPP1R3C*.

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JB Review

DNA methylation accumulation and its predetermination of future cancer phenotypes

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Aberant DNA methylation is a common epigenomic alteration in carcinogenesis. Comprehensive analyses of DNA methylation have stratified gastrointestinal cancer into several subgroups according to specific DNA methylation accumulation. In gastric cancer, *Helicobacter pylori* infection is a cause of methylation accumulation in apparently normal mucosa. Epstein–Barr virus infection is another methylation inducer that causes more genome-wide methylation, resulting in the formation of unique epigenotype with extensive methylation. In colorectal carcinogenesis, accumulation of high levels of methylation in combination with *BRAF* mutation is characteristic of the serrated pathway, but not of the adenoma-carcinoma sequence through conventional adenoma. In a *de novo* pathway, laterally spreading tumours generate intermediate- and low-methylation epigenotypes, accompanied by different genetic features and different macroscopic morphologies. These methylation epigenotypes, with specific genomic aberrations, are mostly completed by the adenoma stage, and additional molecular aberration, such as *TP53* mutation, is suggested to lead to cancer development with the corresponding epigenotype. Accumulation of DNA methylation and formation of the epigenotype is suggested to occur during the early stages of carcinogenesis and predetermines the future cancer type.

Keywords: colorectal cancer/CpG island methylator phenotype (CIMP)/DNA methylation/epigenetics/gastric cancer.

Abbreviations: CIMP, CpG island methylator phenotype; EBV, Epstein–Barr virus; HME, high-methylation epigenotype; IME, intermediate-methylation epigenotype; LME, low-methylation epigenotype; LST, laterally spreading tumour; PRC, Polycomb repressive complex.

Epigenomic information is conveyed by modifications to genomic DNA, such as DNA methylation and histone modification, and impacts cellular behavior by regulating chromatin status and gene expression. Malignant tumours arise through the accumulation of genomic alterations as well as epigenomic changes (*1*, *2*). Epigenomic alterations can accumulate in precancerous, apparently normal tissues to modify cancer risk (*3*, *4*); therefore, they play causal roles not only in cancer progression but also in cancer initiation (*5*, *6*).

Methylation of genomic DNA in mammalian cells is the covalent addition of a methyl group to the cytosine at CpG dinucleotides, and is one of the most important epigenomic modifications (*7*). Although CpG sites in most genomic regions are methylated in normal cells, CpG sites present in the regions containing high numbers of CpG dinucleotides, so-called CpG islands, are generally unmethylated. CpG islands overlap the promoter regions of 60–70% of genes, and are generally protected from methylation, allowing for the expression of downstream genes whose transcription is further regulated by histone modification (*1*).

Aberrant DNA methylation of promoter CpG islands is one of the most important epigenomic alterations of the carcinogenic process that leads to inactivation of gene expression. Several methods to search for aberrantly silenced genes in cancer using promoter methylation as a marker have been developed since the 1990s (*8*). Recent comprehensive DNA methylation analyses have revealed the existence of several cancer subtypes with distinct epigenomic features (*9*, *10*). Infection of different pathogens seems to cause distinct patterns of methylation accumulation, resulting in the formation of cancer with distinct methylation epigenotypes. Colorectal cancer is classified into three major methylation epigenotypes, which strongly associate with unique genetic aberrations and different early lesion morphologies, suggesting unique carcinogenic pathways for each epigenotype (*9*, *11*, *12*). Methylation accumulation is mostly completed by the adenoma stage, and additional molecular aberrations are suggested to herald transition to cancer with the corresponding methylation epigenotypes (*9*, *13*).

Here, we review DNA methylation alteration in gastrointestinal cancer. Aberrant DNA methylation is suggested to accumulate during the early stages of carcinogenesis and may predetermine the future cancer phenotype.

Accumulation of DNA Methylation in Non-Cancerous Tissues

Genes showing aberrant methylation in colorectal cancer, such as *ESR1*, *SFRP1* and *MGMT*, are also reported to be methylated in histologically normal colonic epithelium (*14–16*). Although *MGMT* promoter

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methylation was observed in 46% of colorectal cancers, methylation was also detected in 50% of normal mucosa samples adjacent to methylated tumours, but 6% of those adjacent to unmethylated tumours and 12% of control normal mucosa samples (*16*). This suggested that a proportion of colorectal cancers may arise from mucosa that has been epigenetically modified by *MGMT* promoter methylation. The *SFRP* genes, which encode negative regulators of the WNT signalling pathway and are frequently hypermethylated in colorectal cancer, were also methylated in the normal mucosa of colorectal cancer patients, and at a higher incidence compared with that in patients without colorectal cancer (*15*).

The concept of '*field cancerization*' has been proposed to explain the increased susceptibility of normal tissue to malignant transformation, and was first described in the genesis of oral cancers (*17*). The simultaneous occurrence of multifocal primary lesions and the frequent development of recurrent tumours of the same tissue in nearby locations were also explained using this concept for the upper aerodigestive tract, the oesophagus and the urinary tract (*18–20*). Although genetic alteration is often used to describe the formation of these fields, aberrant DNA methylation in normal, adjacent tissues could be a potential biomarker for assessing the future risk for cancer development. Indeed, the epigenetic field defect has been proposed to occur in colon mucosa as well as in other tissues including, stomach, liver, oesophagus and lung (*16*, *21–24*).

Chronic inflammation is well known to induce aberrant DNA methylation. This has been demonstrated by analyses of non-cancerous tissues, such as colon with ulcerative colitis, liver with chronic hepatitis, oesophagus with inflammatory reflux oesophagitis and stomach with chronic gastritis (*22*, *25–27*). In a mouse model of colitis using dextran sodium sulphate, methylation of CpG islands was induced in colonic epithelial cells within 8 weeks. This methylation increased until CRC development at 15 weeks. The same level of aberrant DNA methylation was also observed in severe combined immunodeficiency mice lacking functional T and B lymphocytes, but was not induced in cultured colon epithelial cells exposed to dextran sodium sulphate. It was suggested that inflammation triggered by dextran sodium sulphate was responsible for methylation induction, but that functional T and B lymphocytes were not essential (*28*).

Ageing is another factor that can promote the accumulation of DNA methylation. For example, *ESR1* was reported to be methylated in histologically normal colonic epithelium in an age-dependent manner (*14*). When newborn and centenarian genomes were compared by whole-genome bisulfite sequencing, centenarian genomes showed decreased DNA methylation throughout the genome, but increased levels of DNA methylation in promoters with a high CpG content (*29*). Another report showed that the epigenome of replicative senescent human cells was similar to the epigenome of cancer, including genome-wide DNA hypomethylation and focal hypermethylation (*30*).

Pathogen infection is associated with a large proportion of human cancers, such as *Helicobacter pylori* with gastric cancer, Epstein–Barr virus (EBV) with lymphoma and other cancers, and hepatitis B and C viruses with liver cancer. These infections can also cause aberrant methylation. *Helicobacter pylori* infection induces DNA methylation accumulation in the gastric mucosa, leading to an epigenetic field defect and a high risk of gastric cancer development (*31*, *32*). EBV infection induces extensive DNA methylation in gastric epithelial cells over a short time period, which can develop into gastric cancer with a unique methylation epigenotype (*10*, *33*). The involvement of these pathogens in gastric cancer is described in next section.

Accumulation of DNA Methylation in Gastric Cancer

High and low levels of methylation in gastric cancer

A subset of cancer cases show significant enrichment of aberrant DNA methylation in specific CpG islands and this phenomenon was first described in colorectal cancer. It was named CpG island methylator phenotype (CIMP), and these CpG islands were established as methylation markers to detect CIMP in colorectal cancer (*34*). Using these CIMP markers for CRC, Toyota *et al.* (*35*) analysed the methylation status of gastric cancer, and suggested that CIMP also exists in gastric cancer, associated with the methylation of *p16* and *MLH1*. In 2002, Kaneda *et al.* conducted a genome-wide search for aberrantly methylated regions in gastric cancer, and identified promoter CpG islands, including some in tumour-suppressor genes, that were aberrantly methylated in gastric cancer (*36*, *37*). Using these promoter CpG islands (for example, in the *LOX* and *THBD* genes) as methylation markers for gastric cancer, the study confirmed the existence of at least two subsets of gastric cancers, with and without unusual accumulation of aberrant promoter methylation (*36*).

Whereas oncogene mutations significantly correlated to methylation accumulation in colorectal cancer and other cancers such as papillary thyroid cancer (*9*, *38*), genetic alterations accompanied with high methylation accumulation have not been clarified well in gastric cancer. Recent comprehensive analyses reported that cancer-related pathways were more frequently affected by aberrant DNA methylation than genetic alteration (*39*), but high-methylation gastric cancer tended to have mutation of oncogenes including *CTNNB1*, *ERBB2*, *KRAS* and *PIK3CA* (*40*).

Methylation induction by H. pylori infection

Helicobacter pylori is a helix-shaped Gram-negative bacterium that has infected the stomachs of approximately half of the world's population (*41*, *42*). An association between aberrant DNA methylation in gastric mucosa and *H. pylori* infection was first shown for *CDH1* methylation (*31*). CDH1 is a cell-to-cell adhesion glycoprotein, frequently inactivated by somatic and germline mutations in sporadic and familial diffuse-type gastric cancers (*43*). Chan *et al.* (*31*) observed promoter methylation of *CDH1* more

frequently in *H. pylori*(+) gastric mucosa than in *H. pylori*(−) mucosa. In 2006, Maekita *et al.* (*27*) performed quantitative methylation analysis on the promoter CpG islands methylated in gastric cancer (*36*), and observed significantly higher methylation levels in *H. pylori*(+) gastric mucosa than in *H. pylori*(−) mucosa. This indicated that promoter CpG islands that are highly methylated in gastric cancer might be associated with *H. pylori* infection and could develop during chronic inflammation.

Eradication of *H. pylori* caused the methylation levels of these markers and other genes to decrease, suggesting that *H. pylori* infection has a causal role in induction of aberrant methylation (*27*, *44*). In a Mongolian gerbil model, 5-aza-2′-deoxycytidine treatment suppressed aberrant DNA methylation, and reduced the incidence of *H. pylori*-induced tumours (*45*). Because this demethylating reagent did not fully prevent the incidence of tumours, the influence of *H. pylori* on factors other than DNA methylation, such as injection of CagA protein into gastric epithelial cells through type IV secretion systems, is also likely to be important in the tumourigenic process (*45–47*). But the induction of aberrant methylation by *H. pylori* seems to contribute, at least in part, to the genesis of gastric cancer.

Although the type IV secretion system enables *H. pylori* to inject factors into gastric epithelial cells, direct roles of *H. pylori* in the induction of aberrant methylation have not been well demonstrated. Rather, indirect mechanisms through chronic inflammation may be involved (*48*). Levels of cytokines, such as IL-1β and TNF, are increased in human ulcerative colitis and hepatitis, and they may be associated with the induction of aberrant DNA methylation during chronic inflammation (*49*, *50*). A single-nucleotide polymorphism of *IL-1β* associates with a high risk of gastric cancer and a high incidence of *CDH1* methylation, whereas IL-1 receptor type 1 knockout mice show decreased levels of DNA methylation in *H. pylori*-induced inflammatory mucosa, indicating that IL-1β-associated inflammation may play a significant role in aberrant methylation induction in chronic gastritis (*51–53*).

Another DNA methylation epigenotype of gastric cancer

Although the majority of gastric cancer is associated with *H. pylori*, a subset of gastric cancer, ranging between 7% and 15%, is associated with EBV infection, a gamma-herpes virus consisting of 184 kb of double-strand DNA (*54*, *55*). Kang *et al.* and others reported that aberrant promoter methylation is involved more frequently in EBV(+) gastric cancer than in EBV(−) gastric cancer. However, although EBV(+) gastric cancer showed a high-methylation phenotype, methylation of *MLH1* was less frequent in EBV(+) cancer (*56*, *57*). Although the genes analysed in these studies were limited to known cancer-associated genes, these results might suggest a unique phenotype of aberrant methylation for EBV(+) gastric cancer.

Matsusaka *et al.* (*10*) performed a comprehensive analysis of DNA methylation in gastric cancer using Infinium bead array technology, and identified three distinct DNA methylation epigenotypes: low-methylation epigenotype (LME), high-methylation epigenotype (HME) and very HME. Low- and high-methylation gastric cancers were all EBV(−) cases, and in good agreement with previous reports (*35*, *36*), EBV(−)/high-methylation gastric cancer involved frequent methylation of *MLH1*. Interestingly, very high-methylation cases were all EBV(+), and never involved *MLH1* methylation.

Methylation marker genes could also be classified into three groups: common, high and EBV+ markers. The common markers were not methylated in normal gastric mucosa, but were aberrantly methylated in all/most gastric cancers regardless of epigenotype. The high-methylation markers were genes aberrantly methylated in both HME and very HME, but not in LME (*10*). The previous methylation marker genes, such as *LOX* and *THBD* (*27*, *36*), were classified as high-methylation markers, suggesting that methylation of these genes might be caused by *H. pylori* infection. Polycomb repressive complex (PRC)-target genes in embryonic stem (ES) cells were highly enriched among the common and high-methylation markers, indicating that PRC-target genes in ES cells could be preferentially methylated in gastric cancer as well as in other cancers (*10*, *58*). Overall, genes methylated in HME are also methylated in EBV(+)/very high-methylation cases. *MLH1* is an exceptional gene that is methylated preferentially in HME, and is never methylated in very HME.

EBV+ markers were never methylated in normal mucosa or in EBV(−) cancer cases, but were specifically methylated in all the very high-methylation gastric cancer cases with EBV infection. Interestingly, EBV+ markers did not show enrichment for PRC-target genes in ES cells, indicating that aberrant methylation is not limited to PRC-target genes in ES cells, but is expanded to non-PRC-target genes in EBV(+) cancer. This suggests that methylation mechanism(s) different from that in EBV(−) cancer, *e.g. H. pylori* infection, should exist (*10*).

EBV infection is the cause of extensive methylation

Using an *in vitro* EBV infection model (*59*), EBV infection itself has been shown to induce aberrant promoter CpG island methylation in a low-methylation gastric cancer cell line, with the methylation dense enough to cause gene repression (*10*). It is notable that not only EBV+ marker genes, but also high-methylation marker genes were also hypermethylated by EBV infection, indicating that the EBV(+)/very HME can be established by EBV infection only, and does not require other causal factors, such as *H. pylori* infection. The fact that *MLH1* is never methylated in clinical EBV(+) cancer nor in EBV-infected cell lines also suggested that factors to methylate *MLH1* and high-methylation marker genes are not involved in the establishment of EBV(+)/very HME.

Helicobacter pylori infection causes chronic inflammation and may lead to the accumulation of aberrant methylation in non-cancerous mucosa over a long time period to form field cancerization (*32*), whereas

methylation by EBV infection may contribute to gastric carcinogenesis in different ways. EBV infection and accumulation of very high methylation is not observed in surrounding non-cancerous mucosa of EBV(+) gastric cancer (*10*, *60*). However, all EBV(+) gastric cancer cases show extensive methylation, and all cancer cells are infected with monoclonal EBV (*10*, *61*). In a case report, gastric dysplasia with EBV infection and without *H. pylori* infection became EBV(+) gastric cancer within several weeks and involved hypermethylation of *CDH1* (*62*). These observations suggest that when methylation is induced by EBV infection, the methylation may expand to silence tumour suppressor genes, which may lead, not to the formation of a field with higher tumour risk, but to cancer formation over a short time period (*33*). *Helicobacter pylori* and EBV thus cause aberrant methylation in completely different ways, resulting in cancer with different phenotypes.

The molecular mechanisms that induce aberrant methylation following EBV infection have not been fully clarified, but some latent genes might contribute to promoter methylation. In a nasopharyngeal cancer cell line and in a gastric cancer cell line, LMP1 and LMP2A, respectively, induced over-expression of DNA methyltransferases and repressed genes such as *CDH1* and *PTEN* (*63*, *64*). The promoter regions of these repressed genes were shown by methylation-specific polymerase chain reaction to be hypermethylated, at least partially (*64*, *65*). It was suggested that latent genes might potentially activate DNA methyltransferases and trigger promoter methylation, although LMPs are not expressed in Burkitt's lymphoma, which is also EBV(+) and shows extensive methylation in the host genome (*33*). Induction of dense promoter methylation has not been demonstrated in latent gene over-expression models (*63–65*), whereas dense methylation is observed in clinical gastric cancer samples and in an *in vitro* EBV infection model (*10*), suggesting that factors other than latent genes are necessary to complete the dense and extensive methylation. The methylation status of viral and host genomes is quite similar in each viral latency programme; aberrant methylation induction was not observed in either viral or host genomes in lymphoblastoid cell lines with type III latency, but extensive hypermethylation was observed in both viral and host genomes in tumour cells with type I or II latencies, such as gastric cancer, Burkitt's lymphoma and nasopharyngeal carcinoma (*33*, *66*). These similarities of methylation status between viral and host genomes suggest that the methylation mechanism of host cells to silence the EBV genome might be uniquely driven in latent I or II infection, which may lead to extensive methylation of the viral and host genomes.

Accumulation of DNA Methylation in Colorectal Cancer

CIMP-positive colorectal cancer

In 1999, Toyota *et al.* identified 33 aberrantly methylated CpG islands in a colorectal cancer cell line, but not in normal colon samples. Most of these CpG islands showed aberrant methylation in an age-related manner, such that they were methylated in aged colon mucosa and most colorectal cancers. Seven CpG islands, however, were not methylated in normal mucosa, and were preferentially methylated in a subset of colorectal cancer cases. These cases were regarded as CIMP(+), and these CpG islands were used as CIMP markers for colorectal cancer (*34*). CIMP involves promoter hypermethylation of the mismatch repair gene, *MLH1*; CIMP therefore associates with microsatellite instability and inversely correlates with chromosome instability (*67*, *68*). CIMP(+) colorectal cancer strongly correlates with *BRAF* mutation, and is preferentially observed in the proximal colon and in women, suggesting existence of a unique carcinogenic pathway different from that in CIMP(−) colorectal cancer (*69*).

Another methylation epigenotype of colorectal cancer

Using the original and other latterly established CIMP markers, Ogino *et al.* proposed a new subset of colorectal cancer, named CIMP-low, with low levels of CIMP marker methylation, other than CIMP-high and CIMP-0 with high and rare methylation of CIMP markers, respectively. CIMP-low could be associated with microsatellite-stable cancer, the male sex and *KRAS* mutations (*70*). A difference between CIMP-low and CIMP-0 was unclear; therefore, another set of methylation markers, ideal for CIMP-low detection, was proposed to be developed (*71*). Using DNA methylation data of 27 previously reported markers and genetic alterations such as mutations of *BRAF*, *KRAS* and *p53*, Shen *et al.* classified colorectal cancer into three subsets with distinct genetic and epigenetic features. The subsets were CIMP1 with *BRAF* mutation and high-methylation, CIMP2 with *KRAS* mutation and different levels of methylation, and CIMP-negative with *p53* mutation and the absence of methylation (*11*).

Yagi *et al.* (*9*) successfully established novel methylation markers through a genome-wide methylation search based on MeDIP-chip analysis, and classified colorectal cancer into three distinct epigenotypes by hierarchical clustering using methylation information alone. HME was strongly correlated with *BRAF* mutation and microsatellite instability, and intermediate-methylation epigenotype (IME) was strongly correlated with *KRAS* mutation. Patients with IME *KRAS*-mutation(+) cancer showed a significantly worse prognosis. *TP53* mutation was absent in HME, but was also detected in both IME and LME. These results were different from those of Shen *et al.* (*11*). These three epigenotypes with distinct genetic and clinicopathological features suggested that there are at least three different molecular pathways in colorectal carcinogenesis (*9*). The existence of these three DNA methylation epigenotypes was confirmed by a subsequent Infinium bead array study (*12*).

For the methylation marker genes, Group-1 markers included most of the known CIMP markers that showed methylation specifically in HME/CIMP-high colorectal cancer. Group-2 markers included many novel methylation genes and showed methylation in

both HME/CIMP-high and IME/CIMP-low. There was no marker that was specifically methylated in IME/CIMP-low. There are some genes commonly methylated in HME/CIMP-high, IME/CIMP-low and LME/CIMP-0, which could not be used as classifier marker genes, but which might be useful as detection markers of colorectal cancer (*9*, *72*).

HME and the serrated pathway

Although the major pathway for colorectal cancer development is considered to be the adenoma-carcinoma sequence from conventional adenoma (*73*), the serrated pathway is an alternative, distinct pathway involving *MLH1* methylation and thus microsatellite instability (*74*). Serrated adenomas commonly show CIMP-high/HME and carry a *BRAF* mutation (*13*, *74*, *75*), whereas these epigenetic and genetic alterations are rarely observed in non-serrated, conventional adenomas (*13*, *76*). Since CIMP-high/HME serrated adenomas and CIMP-high/HME colorectal cancers show similar clinicopathological factors, serrated adenoma is considered as a precursor of CIMP-high/HME cancer (*76*). Methylation accumulation is mostly completed by the adenoma stage, whereas *MLH1* methylation is observed less frequently in serrated adenoma compared with CIMP-high cancer (*76*, *77*). *MLH1* methylation was more frequently observed in large serrated adenomas (*76*), suggesting that *MLH1* methylation is a late event in the serrated pathway, which may contribute to the transition from serrated adenoma to cancer. This is in good agreement with the report that microsatellite instability occurs in developing from adenoma to cancer; copy number alteration was also considered to contribute to cancer development (*78*).

IME and LMEs in early lesions

Yagi *et al.* (*13*) epigenotyped conventional adenoma into two distinct epigenotypes: IME/CIMP-low and LME/CIMP-0; the study did not detect a HME/CIMP-high subset with Group-1 marker methylation in conventional adenoma. IME adenomas were significantly correlated with *KRAS* mutation, similar to colorectal cancer. Interestingly, there was no increase in the methylation level from IME adenoma to IME cancer, suggesting that accumulation of aberrant DNA methylation and its correlation with *KRAS* mutation is already completed by the adenoma stage, and that additional aberrations are needed for adenomas to transform into CRC (*13*).

Sakai *et al.* (*79*) recently found that IME and LME are also involved in the pathology of flat, early colorectal neoplasms. These flat lesions are characterized by lateral extensions along the luminal wall with a low vertical axis, and tumours more than 10 0mm in diameter are called laterally spreading tumours (LSTs). LSTs frequently show high-grade dysplasia, and rapidly develop submucosal invasion despite their small size when accompanying central depression. These neoplasms are considered to develop through a *de novo* pathway, different from the adenoma-carcinoma sequence and the serrated pathway (*80*, *81*). Although IME and LME conventional adenomas did not show remarkable differences in morphological or pathological features, IME and LME in LSTs were detected in lesions with clear morphological differences as below.

There are two types of LST based on macroscopic morphology: granular-type LST and non-granular type LST (*82*). Hiraoka *et al.* (*83*) reported that granular-type LST showed frequent methylation of CIMP markers and frequent *KRAS* mutation. Sakai *et al.* epigenotyped 108 LSTs using quantitative methylation data from pyrosequencing analysis of Group-1 and Group-2 methylation markers. Methylation of Group-1/CIMP markers and *BRAF* mutations was rarely detected in LSTs, suggesting that HME/CIMP-high is unlikely to be involved in LST development. LSTs were clearly clustered into the IME/CIMP-low subset with frequent Group-2 markers, and into the LME/CIMP-0 subset with less frequent Group-2 methylation. Interestingly, IME mostly reflected LST with granular morphology, and was strongly correlated with *KRAS* mutation, while lacking CTNNB1 activation. LME mostly reflected LST with non-granular morphology, a lack of *KRAS* mutation, but significant association with CTNNB1 activation (*79*).

In LSTs with both epigenotypes, methylation levels were not changed between adenoma and cancer, but *TP53* mutations were strongly associated with development from adenoma to cancer (*79*). This suggested that while HME/CIMP-high colorectal cancer develops through a unique carcinogenic pathway (the serrated pathway) showing unique early lesions (serrated adenoma), IME and LME colorectal cancers can also develop through unique pathways with early lesions of different morphologies (*76*, *79*). In each pathway, methylation epigenotype formation and its correlation to unique genetic features appears to be completed at an early stage, at the latest by the adenoma stage (*13*, *79*).

Different types of methylation accumulation and oncogene mutation

It is not known why oncogene mutations correlate with unique methylation epigenotypes. There is a possibility that each mutated oncogene may cause aberrant DNA methylation in a different way. But Hinoue *et al.* (*84*) reported that expression of mutated *BRAF* did not induce any apparent increase of DNA methylation in a CIMP-associated CpG site. In our epigenomic analysis during *RAS* activation, mutated *RAS* in normal cells induced dynamic alteration to histone modification and cellular senescence, but did not alter DNA methylation (*85*). In clinical samples, *KRAS* mutation was frequently observed in aberrant crypt foci, very early colorectal lesions with cellular expansion (*86*). But CIMP was less frequently observed in aberrant crypt foci (*87*), and quantitative methylation analysis showed significantly lower levels of aberrant DNA methylation in all analysed Group-1 and Group-2 markers compared with adenoma (*13*). There seems to be no causal relationship between oncogene mutation and DNA methylation induction. Tumorous cell growth to form adenoma/cancer might require the independent and simultaneous occurrence of aberrant

DNA methylation with oncogene mutation, whereas oncogene mutation without DNA methylation accumulation might result in the limited cell growth seen in premature cell senescence or aberrant crypt foci.

It is also not known how aberrant methylation is accumulated differently in the colon. Chronic inflammation or ageing could be the cause of methylation accumulation (*14*, *25*), but this does not explain how the different methylation accumulation patterns are constituted in distinct methylation epigenotypes. As different pathogens induce different methylation patterns in gastric carcinogenesis (*32*, *33*), some pathogens might perhaps be involved in epigenotype formation in colorectal carcinogenesis. In other types of cancer such as glioma and acute myeloid leukaemia, *IDH1/2* mutation correlates significantly with high-methylation accumulation (*88*, *89*), and *IDH1* mutation was the cause of aberrant methylation induction (*89–91*). TET family proteins are considered to play an important role in DNA demethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine (*92*). TET2 was frequently mutated in acute myeloid leukaemia in mutually exclusive manner with *IDH1/2* mutation, and mutated IDH1 inhibited TET2-dependent hydroxylation of 5-methylcytosine (*89*, *90*). Whereas TET2 mutation was reported to involve methylation changes outside gene promoters (*93*), TET inactivation may somehow function in aberrant methylation accumulation, as well as *CDH1/2* mutation. In colorectal cancer, *BRAF* and *KRAS* mutations are not considered to induce aberrant DNA methylation, but other unknown genetic aberration(s) might perhaps cause methylation accumulation to form CIMP-high/HME or CIMP-low/IME. Although the mechanism of specific epigenotype formation has not been identified, methylation accumulation occurs at an early stage of colorectal carcinogenesis, with unique epigenotype formation completed by the adenoma stage (*13*, *79*). These early lesions with specific epigenotypes are considered as the precursors of cancers with the corresponding epigenotypes.

Conclusions

Cancer can be classified into several phenotypes of aberrant DNA methylation. Each epigenotype exhibits distinct clinicopathological features, including associated genetic aberrations, morphologies, locations, sex, prognosis and pathogens. Aberrant DNA methylation can be accumulated in apparently normal tissues, and the corresponding methylation epigenotypes with unique features are observed in both adenoma and cancer. DNA methylation accumulation to form unique epigenotypes is suggested to be an early event in carcinogenesis, and thus may predetermine future cancer phenotypes. Determining the detailed molecular mechanisms of methylation induction will lead to further understanding of carcinogenic pathways, and to the establishment of molecular targets for cancer risk therapy.

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Conflict of Interest

None declared.

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Chapter 15

Quantitative DNA Methylation Analysis for Epigenotyping of Colorectal Cancer

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Abstract

Accumulation of epigenetic alteration plays important roles in tumorigenesis. Aberrant DNA hypermethylation in gene promoter regions is a common epigenetic mechanism for silencing tumor suppressor genes in many types of cancer including colorectal cancer (CRC). By using quantitative methylation information, CRC can be classified into three distinct methylation epigenotypes with different genetic features, suggesting existence of at least three molecular pathways in genesis of CRC. We describe in this chapter, the methods for analyses of aberrant DNA methylation to epigenotype CRC.

Key words Colorectal cancer, DNA methylation, Epigenotype, MALDI-TOF-MS, CpG island methylator phenotype (CIMP)

1 Introduction

Cancer arises through accumulation of genetic alteration and epigenetic alteration [1–3], including colorectal cancer (CRC) [4]. Frequent mutations of genes, such as *KRAS*, *p53*, and *APC*, were detected in CRC; epigenetic alteration such as DNA methylation or loss of imprinting also plays an important role in colorectal carcinogenesis, and aberrant hypermethylation of gene promoter regions is a common epigenetic mechanism for gene silencing involved in the initiation and progression of cancer [5–9]. In 1999, a subgroup of CRC was reported to show significantly frequent CpG island methylation, the so-called CpG island methylator phenotype (CIMP) [10]. CIMP + CRC significantly correlates to microsatellite instability and *BRAF* mutation [11].

In our previous studies, we epigenotyped colorectal cancer and adenoma by two-way hierarchical clusteting method using highly

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quantitative DNA methylation data, and identified three clusters of colorectal cancer and adenoma with distinct methylation epigenotypes [6, 12]. High-methylation epigenotype correlated to *BRAF*-mutation(+) and microsatellite instability [6], as CIMP was previously reported [11]. Serrated adenoma showed this epigenotype, indicating that high-methylation CRC arises through serrated pathway [12–14]. In microsatellite-stable colorectal cancer, intermediate-methylation epigenotype correlated to *KRAS*-mutation(+) and lack of *BRAF* mutation, and low-methylation epigenotype correlated to lack of *BRAF/KRAS* mutation. Conventional, non-serrated adenoma was clustered into these two epigenotypes, indicating that intermediate- and low-methylation cancers arise through conventional adenomas [12].

DNA methylation markers were also clustered into two groups, Group-1 and Group-2 markers [15]. Group-1 markers included most of previously established CIMP markers [10, 11, 16], and are methylated specifically in high-methylation/CIMP+colorectal cancer. Group-2 markers are methylated in both high- and intermediate-methylation epigenotypes, but not in low-methylation epigenotype. Therefore, CRC with methylation of both Group-1 and Group-2 markers is regarded as high-methylation, and CRC without Group-1 marker methylation but with methylation of Group-2 markers is regarded as intermediate-methylation epigenotype [6, 15].

We here introduce the methods to analyze the methylation markers quantitatively, by bisulfite-PCR based highly quantitative method, MALDI-TOF mass spectrometry (MassARRAY®).

2 Materials

2.1 Clinical Samples

Tissue samples of clinical colorectal cancer, adenoma, and normal mucosa are obtained with written informed consents and with approval by Ethics Committee of the institutes.

1. Keep tissues at −80 °C until use.
2. Embed frozen tissues in OCT medium, and slice them using Criostat.
3. Stain a 5 μm-thick specimen with Giemsa and microscopically examine.
4. When the tumor cell content is at least 40 %, collect 10 consecutive 10 μm-thick slices and keep at −80 °C. Stain a slide with hematoxylin–eosin (H/E) (*see* **Note 1**).
5. The H/E slides are microscopically examined for determination of tumor cell contents by two independent pathologists. When the tumor cell content is at least 40 %, the samples are selected for further study (*see* **Note 2**).