

Intermediate Methylation Epigenotype and Its Correlation to *KRAS* Mutation in Conventional Colorectal Adenoma

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A subset of colorectal cancer shows significant accumulation of aberrant promoter methylation. Previously, we developed two groups of methylation markers that classified colorectal cancer into three epigenotypes: i) high-, ii) intermediate-, and iii) low-methylation epigenotypes. High-methylation epigenotype, with methylation of both group 1 and group 2 markers, correlates to *BRAF*-mutation(+). Intermediate-methylation epigenotype, with methylation of group 2 markers, but not group 1, correlates to *KRAS*-mutation(+). To gain insight into epigenotype development in colorectal carcinogenesis, especially intermediate-methylation epigenotype and its correlation to *KRAS*-mutation(+) in adenoma, we analyzed methylation levels of group 1 and group 2 markers quantitatively by matrix assisted laser desorption ionization-time of flight mass spectrometry, in 51 adenomas, 13 aberrant crypt foci, and 26 normal mucosa samples, and we compared them to 149 previously analyzed colorectal cancer samples. Three serrated adenomas were all *BRAF*-mutation(+), showing great methylation of group 1 and group 2 markers, thus high-methylation epigenotype. Forty-eight conventional adenomas were not methylated in group 1 markers and were classified into two clusters with higher and lower methylation of group 2 markers, thus into intermediate- and low-methylation epigenotypes, respectively. Ade-

noma with intermediate-methylation epigenotype correlated to *KRAS*-mutation(+). Methylation levels of group 2 markers in adenoma were higher than aberrant crypt foci and normal samples, but similar to cancer. These data suggested that epigenotype development occur at an earlier stage than carcinoma formation, and already be completed at the adenoma stage. Intermediate methylation epigenotype and its correlation to *KRAS*-mutation(+) were developed in conventional adenoma. (*Am J Pathol* 2012, 180:616–625; DOI: 10.1016/j.ajpath.2011.10.010)

Colorectal cancer arises as a consequence of genetic alteration and epigenetic alteration.¹ Gene mutations (eg, *KRAS*, *p53*, and *APC*) are well-known genetic alterations that occurred in colorectal cancer, which were demonstrated in the model of “adenoma-carcinoma sequence” by Bert Vogelstein.² Epigenetic alteration, such as DNA methylation or loss of imprinting, is also important in colorectal carcinogenesis, and aberrant promoter methylation is a major epigenetic mechanism for gene silencing to be involved in the initiation and progression of cancer.^{3,4} As for accumulation of aberrant methylation, Toyota et al⁵ reported in 1999 that a subset of colorectal cancer shows significantly frequent CpG island methylation [ie, the so-called CpG island methylator phenotype (CIMP)]. CIMP⁺ colorectal cancer significantly correlates to microsatellite instability and *BRAF* mutation.⁶

Colorectal adenoma is known as a precursor lesion of colorectal cancer. Serrated adenomas were reported to show CIMP⁺ and frequent *BRAF* mutation, and DNA methylation was thus considered to be an early event in the serrated pathway, which explains carcinogenesis

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from serrated adenoma to colorectal cancer with microsatellite instability.^{7,8} Adenomas without serrated features (nonserrated adenomas) were classified as conventional adenomas, which correspond to tubular, tubulovillous, and villous adenomas.⁹ Existence of methylation phenotype in the conventional adenomas and its correlation to *KRAS* mutation were largely unknown.

In our previous study, we epigenotyped colorectal cancer by the two-way hierarchical clustering method

using highly quantitative DNA methylation data, and we identified three clusters of colorectal cancer with distinct methylation epigenotypes.¹⁰ High-methylation epigenotype (HME) correlated to *BRAF*-mutation(+) and microsatellite instability,¹⁰ as CIMP was previously reported.⁶ In microsatellite stable colorectal cancer, intermediate methylation epigenotype (IME) correlated to *KRAS*-mutation(+) and a lack of *BRAF* mutation, and low methylation epigenotype (LME) correlated to lack of *BRAF/KRAS* mu-

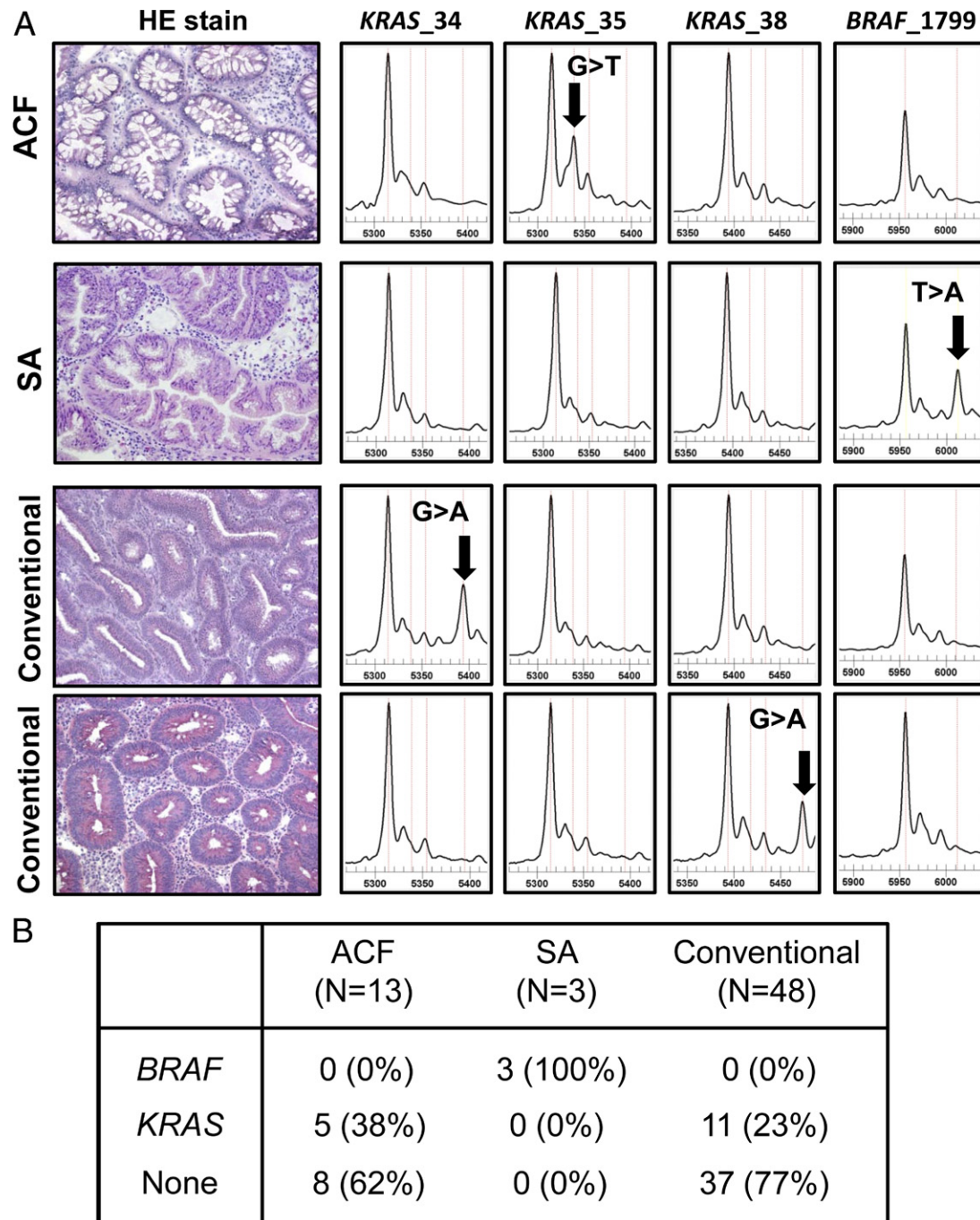


Figure 1. Oncogene mutation statuses in aberrant crypt foci (ACF) and adenoma. **A:** Representative results of H&E staining (×200) and oncogene mutations detected by genotyping assay using matrix assisted laser desorption ionization-time of flight-mass spectrometry. The content of ACF or adenoma cells was confirmed to be more than 40% (H&E staining), so that the peak for a mutant allele (arrow) would be high enough, presumably higher than a quarter of the peak height for wild-type allele. **B:** Summary of oncogene mutation frequency, in which all of the three serrated adenoma samples were *BRAF*-mutation(+), whereas 11 of 48 conventional adenoma samples were *KRAS*-mutation(+). Conventional, conventional adenoma; HE, H&E; SA, serrated adenoma.

Table 1. Methylation Marker Genes and Primer Sequences

Gene symbols	Primer sequences		Positions (TSS = +1)		Group
	Forward	Reverse	Forward 5'	Reverse 5'	
ADAMTS1	5'-GTTTTTTGGGGTTTAAATGT-3'	5'-CTCCRACACCCTAACTCCTC-3'	+440	+647	2
COL4A2	5'-TAGYGTAGGATGAGGGAGGT-3'	5'-CRCCTTATACAACTAAAACCTACAC-3'	+302	+551	2
DFNA5	5'-GGTTAGATTTTTTAGAAGTTTGA-3'	5'-AATCCCACTCCACTATAAATAAC-3'	+170	+435	2
EFEMP1	5'-TAGGAGTTGGTTAGAAGTTGG-3'	5'-ACRACAAATCTCTTTTATCTTATCA-3'	-167	+53	2
ELMO1	5'-AATGTGTTTTTGGTTAGTAGGAG-3'	5'-AAATAACTCTACCTCTATCCTATACC-3'	-89	+61	2
FBN2	5'-GGATATTGGAAAGTTGTAAG-3'	5'-CCRCCTCTCTCTTACTAAC-3'	-176	+13	2
HAND1	5'-GGGAAAGTTTATAGTGGAGAG-3'	5'-CAAATCATCACTCCTTAAAAATC-3'	-1216	-950	2
IGFBP3	5'-GTTTTTTTGGGATTTTATAGTT-3'	5'-AAACAACACCAACAAAATCAAC-3'	+38	+189	2
IGFBP7	5'-GAGAAGGTTATTATTAGGTTAGTAA-3'	5'-ACTACCAACTCTTCCCTCC-3'	+455	+651	2
LOX	5'-TGTATTGTTTGGTGGAGAT-3'	5'-AAACTCAACAACTAAACACCTA-3'	+128	+453	1
MINT31	5'-GGTGGTGTAGTTTATAGGAGAG-3'	5'-AACACTTCCCCAACATCTAC-3'	NA	NA	1
NEUROG1	5'-AGTTTGGGTTGTTATTTTGT-3'	5'-CTTAAAAATCCTAAAACCAATC-3'	-42	+160	2
RUNX3	5'-GAGTAGTGGGATGGGAGGT-3'	5'-CCRTTAAAAATCATTCCTACAAAAC-3'	+771	+902	1
SFRP1	5'-GTTTTGTTTTTTAAGGGGTGTTGAG-3'	5'-ACACTAACTCCRAAACTACAAAAC-3'	-186	+22	2
STOX2	5'-GGTTTTAGGTTGGGGTAGTT-3'	5'-GGTTTTAGGTTGGGGTAGTT-3'	+291	+568	2
THBD	5'-TAGTTTTTTTATATAGGATTTTTTTT-3'	5'-CCCAACATATTACCCAAAC-3'	-99	+169	2
TSPYL5	5'-GGAAGAGATGAAATGGTAGTAT-3'	5'-TCAAAAACACRCTATAACCCTA-3'	-183	+85	2
UCHL1	5'-YGGTAGAAATAGTTTAGGGAAG-3'	5'-TACTCCATACACTCAAAAAACAC-3'	-234	+51	2

Positions of 5' end of the primers were shown, regarding transcription start site as + 1 bp. NA, not applicable, because MINT31 is not a gene.

tation. These three epigenotypes and their strong correlation to different oncogene mutations suggested distinct molecular genesis of colorectal cancer.¹⁰

The two-way hierarchical clustering in our study also classified DNA methylation markers into two groups (ie, group 1 and group 2 markers).^{10,11} Group 1 markers included most of the previously established CIMP markers^{5,6,12} and were characterized to be methylated specifically in HME/CIMP⁺ colorectal cancer. Group 2 markers are methylated in both HME and IME, but not in LME. Therefore, colorectal cancer methylated in group 1 markers (and also inevitably group 2 markers) is regarded as HME, and colorectal cancer without group 1 marker methylation (but methylated in group 2 markers) is regarded as IME.¹¹

Whereas *BRAF* mutation and CIMP marker methylation were reported in serrated adenomas,^{7,8} methylation accumulation in conventional adenomas, or association between the methylation and *KRAS* mutation, has not been clarified yet. We therefore analyzed methylation status in 48 conventional adenomas using 15 group 2 markers, as

well as three group 1 markers, by bisulfite-PCR-based highly quantitative method, matrix assisted laser desorption ionization-time of flight mass spectrometry (MassARRAY). Herein we report that epigenotype development is earlier than cancer stage and already completed at adenoma stage, and that IME and its correlation to *KRAS* mutation are developed in conventional adenoma.

Materials and Methods

Clinical Samples

Clinical colorectal adenoma, aberrant crypt foci, and normal mucosa samples were obtained from the patients who underwent endoscopic examination at Yokohama City University Hospital with written informed consents, and kept at -80°C until use. Colorectal cancer samples were obtained from the patients who underwent surgery at Saitama Cancer Center, with written informed consents, and their DNA was extracted in our previous

Table 2. Primer Sequences Used for Mutation Analysis

Mutation sites	Primer sequences	Extend primers
<i>KRAS</i> _34		
Forward	5'-ACGTTGGATGAGGCCTGCTGAAAATGACTG-3'	5'-ACTCTTGCTTACGCCAC-3'
Reverse	5'-ACGTTGGATGTAGCTGTATCGTCAAGGCAC-3'	
<i>KRAS</i> _38		
Forward	5'-ACGTTGGATGAGGCCTGCTGAAAATGACTG-3'	5'-AGGCACTCTTGCTTACG-3'
Reverse	5'-ACGTTGGATGTAGCTGTATCGTCAAGGCAC-3'	
<i>KRAS</i> _35		
Forward	5'-ACGTTGGATGAGGCCTGCTGAAAATGACTG-3'	5'-CACTCTTGCTTACGCCA-3'
Reverse	5'-ACGTTGGATGTAGCTGTATCGTCAAGGCAC-3'	
<i>BRAF</i> _1799		
Forward	5'-ACGTTGGATGTCTTCATGAAGACCTCACAG-3'	5'-CCCACTCCATCGAGATTTC-3'
Reverse	5'-ACGTTGGATGTTCAAACCTGATGGGACCCAC-3'	

study.¹⁰ All of the aberrant crypt foci and adenoma samples were microscopically examined for determination of its lesion contents by two independent pathologists. Then, 51 adenoma (2 traditional serrated adenomas, 1 sessile serrated adenoma, and 48 conventional adenomas) and 13 aberrant crypt foci samples that contained at least 40% of lesion cells, were prepared (Figure 1A). We analyzed methylation and oncogene mutation statuses of 48 conventional adenoma samples, and compared them with 3 serrated adenomas, 13 aberrant crypt foci, 26 normal mucosa samples, and 149 previously analyzed colorectal cancers.¹⁰ DNA of clinical samples was extracted using QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). This study was certified by the Ethics Committee in Tokyo University, Yokohama City University and Saitama Cancer Center.

Bisulfite Treatment

Bisulfite conversion of genomic DNA was performed as previously described.¹³ After sonication of genomic DNA in 30 seconds by Bioruptor (Cosmobio Co. Ltd., Tokyo, Japan), 500 ng of DNA was denatured in 0.3 N NaOH, and then subjected to 15 cycles of 30 seconds at 95°C and 15-minute incubation in 3.6 M sodium bisulfite and 0.6 mmol/L hydroquinone at 50°C. The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), desulfonated in 0.3 N NaOH at room temperature for 5 minutes, and then purified by ethanol precipitation. Finally, genomic DNA was diluted by 40 μ L of distilled water.

Methylation Analysis

Quantitative methylation analysis was performed using MALDI-TOF mass spectrometry (MassARRAY, Sequenom, San Diego, CA).^{10,14} Bisulfite-treated DNA was amplified by PCR, the PCR product was transcribed by *in vitro* transcription, and the RNA was cleaved by RNaseA. Unmethylated cytidine was converted to Uridine by bisulfite treatment [ie, thymidine in the PCR product], and finally adenosine in the *in vitro* transcription product. Methylated cytidine was not converted (ie, cytidine in the PCR product), and finally guanosine in the *in vitro* transcription product. Since RNaseA cleaves RNA at the 3' site of both thymidine and cytidine, thytidine-specific cleavage was possible by containing deoxycytidine instead of cytidine in the *in vitro* transcription mixture. Methylation status was determined by mass difference between adenine and guanine in a cleaved RNA product. Quantitative methylation was calculated for each cleaved product. This analytic unit containing several CpG sites in a cleaved product was called the "CpG unit."

Primers were designed in the previous study¹⁰ to include no CpG site or only one CpG site in 5' regions of primers, which is listed in Table 1. Three group 1 markers were randomly chosen and used, because three were enough to confirm hypermethylation status of group 1 markers (\equiv CIMP markers) in serrated adenoma. As many as 15 group 2 markers showing a variety of average methylation levels¹⁰ were chosen, because investigation on IME existence in conventional adenomas was the ma-

jor purpose of this study, and such number of markers were necessary for hierarchical clustering and demonstration of methylation development in precursor lesions. All of the primers were validated for their accuracy for quantitative analysis by calculating correlation coefficient (r^2) of the standard curve using methylation control samples (0, 25, 50, 75, and 100% methylation) at each CpG

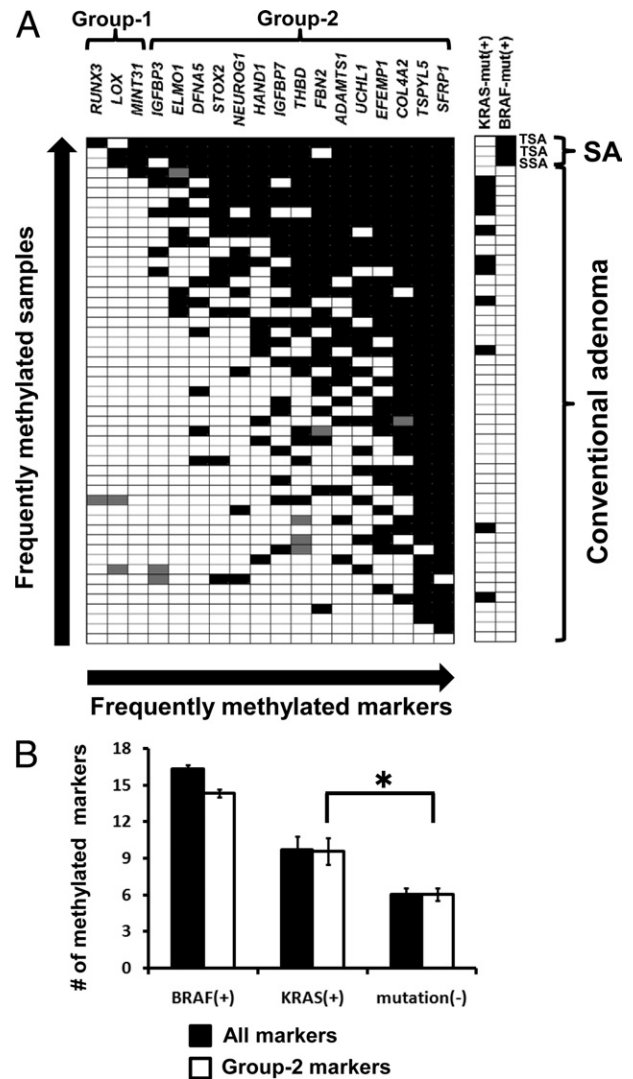


Figure 2. Methylation frequency of 18 markers in adenoma samples. **A:** Methylation was regarded as (+) when methylation rate by quantitative analysis was $>35\%$ (closed box). Gray box indicates no results in matrix assisted laser desorption ionization-time of flight-mass spectrometry. Samples were sorted from top to bottom in descending order for the number of methylated markers. Markers were sorted from left to right in ascending order for the number of methylated samples. The three most frequently methylated samples were serrated adenoma (SA); each sample showed methylation in two of the three group-1 markers, as well as very frequent methylation of group-2 markers (93 to 100%). In 48 conventional adenoma samples, group-1 markers were hardly methylated, and the methylation of group-2 markers varied (0% to 93%). *KRAS* mutation was enriched upward in the panel, and there was significant correlation between frequent methylation of group-2 markers and *KRAS* mutation ($P = 2.7 \times 10^{-3}$, Wilcoxon rank sum test). SSA, sessile serrated adenoma; TSA, traditional serrated adenoma. **B:** Comparison of number of methylated markers. The three *BRAF*-mutation(+) serrated adenoma samples showed markedly frequent methylation. *KRAS*-mutation(+) adenomas showed significantly frequent methylation of group-2 markers (9.7 ± 3.6 markers) than oncogene-mutation(-) adenomas (6.0 ± 3.1 markers; $*P = 7.8 \times 10^{-3}$).

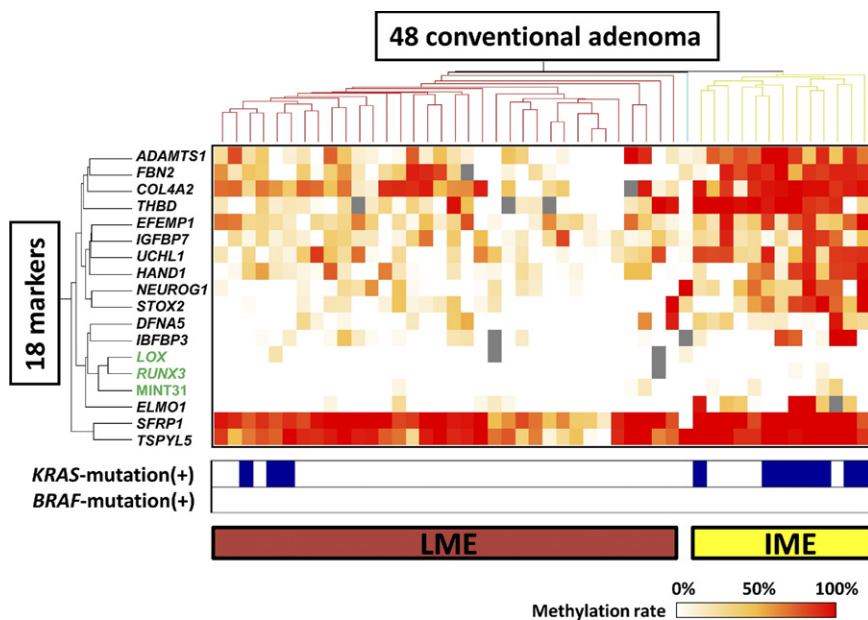


Figure 3. Unsupervised hierarchical clustering of 48 conventional adenoma samples. Markers in black indicate group 2 markers, and markers in green indicate group 1 markers. Conventional adenoma samples were classified into two clusters: the right cluster of 13 cases (yellow) shows higher methylation of group 2 markers and significantly high frequency of *KRAS* mutation ($P = 4.7 \times 10^{-4}$, Fisher's exact test), which was considered to be intermediate methylation epigenotype (IME); the left cluster of 34 cases (red) shows less or no methylation of group 2 markers and less frequency of *KRAS* mutation, which was considered to be low methylation epigenotype (LME). One case (pale blue) was considered as an outlier. As for the markers, group 1 markers were hardly methylated and were grouped into one cluster (green).

unit. CpG units with $R^2 \leq 0.9$ were excluded, and primer pairs whose amplicon contained three or more CpG units with $R^2 > 0.9$ were used in this study.

Mutation Analysis

Mutation at *BRAF* 1799 and *KRAS* 34, 35, and 38 were analyzed by genotyping assay on MassARRAY platform.¹⁵ First, PCR amplification primers and a post-PCR extension primer were designed using MassARRAY Assay Design 3.0 software (Sequenom), as listed in Table 2. *BRAF* 1799 and *KRAS* 38 mutation were analyzed in a single reaction by multiplex PCR. The PCR amplification was performed in 5 μ L volumes con-

taining 0.5 units of Taq polymerase, 5 ng of genomic DNA, 0.5 pmol of PCR primer, and 2.5 nmol of deoxyribonucleotide triphosphates. PCR reactions were cycled at 94°C for 15 minutes, followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute. Shrimp alkaline phosphatase treatment was performed at 37°C for 20 minutes and 85°C for 5 minutes. Post-PCR primer extension was performed using 5.6 pmol of extension primer. Extension reaction were cycled at 94°C for 30 seconds, followed by 40 cycles of 94°C for 5 seconds, 5 cycles of 52°C for 5 seconds, and 80°C for 5 seconds, and 72°C for 3 minutes. Reaction products were transferred to a SpectroCHIP (Sequenom), and mass difference was

Table 3. Clinical and Molecular Characteristics of Adenoma according to Three Epigenotypes

Clinical or molecular features	HME	IME	LME	P value (IME versus LME)
Number of samples	3 (6%)	13 (26%)	34 (68%)	
Sex				
Male	2 (66%)	12 (92%)	23 (68%)	0.14
Female	1 (33%)	1 (8%)	11 (32%)	
Age				
Mean \pm SD	72 \pm 11.1	66.7 \pm 5.6	66.9 \pm 10.5	0.93
Tumor size				
Average (mm) \pm SD	13.3 \pm 2.9	13.6 \pm 5.9	9.7 \pm 4.2	0.043*
>10 mm	2 (66%)	7 (54%)	10 (29%)	0.18
\leq 10 mm	1 (33%)	6 (46%)	24 (71%)	
Tumor location				
Proximal	1 (33%)	3 (23%)	14 (41%)	0.32
Distal	2 (67%)	10 (77%)	20 (59%)	
<i>BRAF</i> mutation				
(+)	3 (100%)	0 (0%)	0 (0%)	1
(−)	0 (0%)	13 (100%)	34 (100%)	
<i>KRAS</i> mutation				
(+)	0 (0%)	8 (62%)	3 (9%)	4.7×10^{-4} *
(−)	3 (100%)	5 (38%)	31 (91%)	

Proximal location is the cecum to the transverse colon. Distal location is the descending colon to the rectum.

*P value between IME and LME < 0.05 (calculated by Fisher's exact test, except age and tumor size by Student's t-test).

HME, high methylation epigenotype; IME, intermediate methylation epigenotype; LME, low methylation epigenotype; SD, standard deviation.

analyzed using MALDI-TOF mass spectrometry, to see the extended base at the possible mutation site.

Statistical Analysis

Correlation between epigenotypes and clinicopathological factors, except age and tumor size were analyzed by Fisher's exact test. Age and tumor size were analyzed by Student's *t*-test. Unsupervised two-way hierarchical clustering was performed based on Euclid distance correlation and average linkage clustering algorithm in sample and marker directions using GeneSpring 7.3.1 software (Agilent Technology, Santa Clara, CA).

Results

Mutation of Adenoma and Aberrant Crypt Foci

We analyzed mutation statuses of *BRAF* and *KRAS* in 51 colorectal adenomas (3 serrated and 48 conventional adenomas) and 13 aberrant crypt foci, using MALDI-TOF mass spectrometry (Figure 1). All of the three serrated adenoma samples (including two traditional and one sessile serrated adenomas) showed *BRAF* mutation (100%) and no *KRAS* mutation (0%). Among 48 conventional adenoma samples, 11 showed *KRAS* mutation (23%) and none showed *BRAF* mutation (0%). In 13 aberrant crypt foci, 5 showed *KRAS* mutation (38%) and none showed *BRAF* mutation (0%).

Methylation Rate of Analyzed Samples

Among group 1 and group 2 markers we established in the previous study,¹⁰ we analyzed methylation rates of three group 1 markers and 15 group 2 markers in adenoma, aberrant crypt foci, and normal mucosa samples.

Methylation frequency of 18 markers in adenoma samples was summarized in Figure 2A. Three top-ranking samples with the most frequent methylation were serrated adenomas; each sample showed methylation in two of the three group 1 markers as well as very frequent methylation of group 2 markers, ranging 93 to 100%. The three serrated adenoma samples were thus considered as HME, and all of the three showed *BRAF* mutation whereas none of conventional adenoma showed *BRAF* mutation ($P = 4.8 \times 10^{-5}$) (Figure 1). In conventional adenoma, group 1 markers were hardly methylated, suggesting that there was no HME case. Group 2 marker methylation varied from 0% to 93% (Figure 2A). There was significant correlation between frequent methylation of group 2 markers and *KRAS* mutation ($P = 2.7 \times 10^{-3}$, Wilcoxon rank sum test).

When number of methylation markers and oncogene mutation status were compared, the three *BRAF*-mutation(+) serrated adenoma samples showed markedly frequent methylation (Figure 2B). *KRAS*-mutation(+) adenoma showed significantly frequent methylation of group 2 markers (9.7 ± 3.6 markers) than *BRAF*/*KRAS*-mutation(-) adenoma (6.0 ± 3.1 markers, $P = 7.8 \times 10^{-3}$, Student's *t*-test).

Hierarchical Clustering of Conventional Colorectal Adenoma

To analyze whether conventional adenoma can be classified into some clusters using DNA methylation information, two-way unsupervised hierarchical clustering method was applied in the analysis using quantitative methylation data (Figure 3). Conventional adenoma was classified into two major clusters; a cluster with higher methylation rate of group 2 markers was considered as IME, and the other with lower methylation rate was LME. IME adenoma showed significant correlation to *KRAS*-mutation(+) ($P = 4.7 \times 10^{-4}$, Fisher's exact test) (Figure 3). As for other clinicopathological factors, adenoma size in IME was significantly larger than LME ($P = 0.043$,

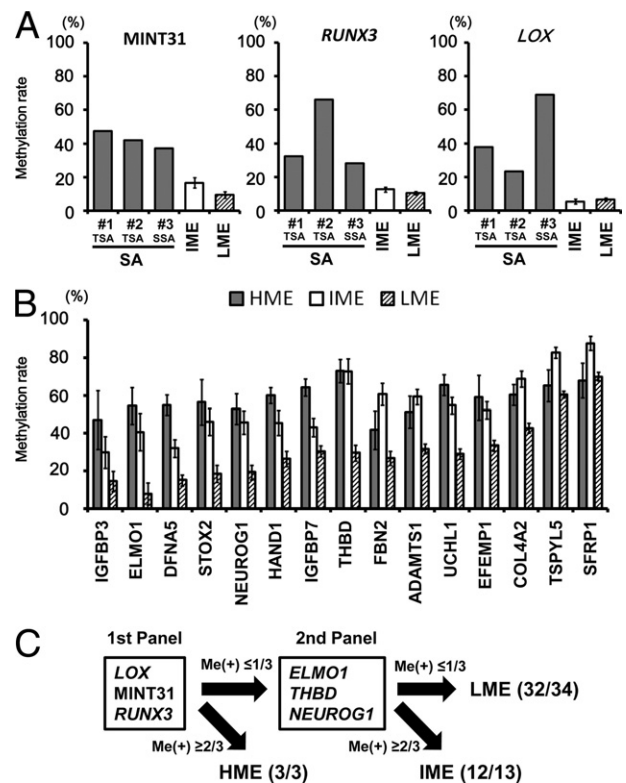


Figure 4. Comparison of methylation rates in different epigenotypes. Methylation levels were shown by average methylation rate \pm SE. **A:** Methylation rate of group 1 markers in three serrated adenomas (SA) (gray box), intermediate methylation epigenotype (IME) conventional adenomas (open box), and low methylation epigenotype (LME) conventional adenoma (hatched box). The three serrated adenomas showed higher methylation rate compared to IME and LME adenomas. These three *BRAF*-mutation(+) serrated adenoma samples were thus considered to be high methylation epigenotype (HME). SSA, sessile serrated adenoma; TSA, traditional serrated adenoma. **B:** Methylation rate of group 2 markers. IME adenoma samples showed a higher methylation rate in all of the group 2 markers compared to LME. Group 2 markers had been classified into H>I>L and H=I>L types in a previous study of colorectal cancer,^{10,11} whereas similar methylation patterns were confirmed in adenoma. For example, *ELMO1*, *STOX2*, *NEUROG1*, *HAND1*, and *IGFBP7* were included in H>I>L type, and *THBD*, *FBN2*, *ADAMTS1*, *EFEMP2*, and *COL4A2* in H = I>L type. **C:** Two-step marker panel. Epigenotypes can be determined using two panels of markers, without hierarchical clustering method. The first panel containing three group 1 markers extracted cases with methylation of ≥ 2 markers as HME. The remaining cases with methylation of ≤ 1 marker undergo the second panel containing three group 2 markers, and were classified into cases with methylation of ≥ 2 markers as IME, and cases with methylation of ≤ 1 marker as LME. Among 50 cases, there were 47 (94%) that were accurately determined.

Student's *t*-test). Tumor location did not show significant difference ($P = 0.31$, Fisher's exact test) (Table 3).

Comparison of Methylation Levels among Different Epigenotypes

The methylation rates of markers were compared according to epigenotypes (Figure 4). In group 1 markers, the three serrated adenoma samples showed higher methylation rate than IME and LME adenomas, confirming that the serrated adenoma is HME (Figure 4A). In most group 2 markers, the methylation rate was generally highest in HME, lowest in LME, and at an intermediate level in IME (Figure 4B). We had classified group 2 markers into $H>I>L$ and $H=I>L$ types in a previous study of colorectal cancer,^{10,11} whereas similar methylation patterns were confirmed in adenoma (eg, *ELMO1*, *STOX2*, *NEUROG1*, *HAND1*, and *IGFBP7* as $H>I>L$ type, and *THBD*, *FBN2*, *ADAMTS*, and *COL4A2* as $H=I>L$ type).

Development of Two-Step Marker Panel

In the previous analysis of colorectal cancer, we developed a two-step panel method to classify HME, IME, and LME easily without hierarchical clustering.¹⁰ Because group 1 markers are methylated specifically in HME, and group 2 markers are methylated commonly in HME and IME, HME cases should be extracted as frequently methylated samples using the first panel containing three to five group 1 markers. The remaining cases could be divided into IME and LME by the second panel containing three to five group 2 markers.¹¹ Here we developed a two-step marker panel in the similar manner, with 94% accuracy (Figure 4C). The first panel containing three group 1 markers (*LOX*, *MINT31*, and *RUNX3*) can extract three cases with methylation of ≥ 2 markers as HME (3/3; 100% accuracy). The remaining 47 cases undergo the second panel containing three group 2 markers (*ELMO1*, *THBD*, and *NEUROG1*). There were 47 cases that were divided into 13 cases with methylation of ≥ 2 markers as IME (12/13; 92% accuracy), and 34 cases with methyl-

ation of ≤ 1 marker as LME (32/34; 94% accuracy); this is an easier and useful decision method than an 18 gene panel (Figure 2A) and hierarchical clustering (Figure 3).

Methylation of Adenoma and Carcinoma

It was shown that methylation is accumulated enough at the stage of adenoma to develop epigenotype with correlation to oncogene mutation. To analyze whether there is any more increase of methylation rate from colorectal adenoma to cancer, average methylation rate was compared between adenoma and cancer samples (Figure 5). In IME adenoma and cancer, there was no difference of methylation rate in any markers from adenoma, to stage II CRC, stage III CRC, and stage IV CRC (Figure 5A). In LME, although *COL4A2* showed a slight but significant increase of methylation rate from adenoma to cancer, there was no difference of methylation rate in any other markers (Figure 5B). It was suggested that accumulation of the aberrant promoter methylation was mostly completed at adenoma stage already, at least for the analyzed markers.

Comparison of Aberrant Crypt Foci and Adenoma

To analyze whether there is any difference of methylation rate in adenoma and any other colorectal lesion with oncogene mutation, the average methylation rate was compared among normal colorectal mucosa, aberrant crypt foci, and adenoma samples (Figure 6). All of the 15 group 2 markers showed significant methylation increase in adenoma compared to normal colorectal mucosa, and also 12 of 15 group 2 markers in adenoma compared to aberrant crypt foci, whereas only *SFRP1* among 15 group 2 markers showed significant methylation difference between normal colorectal mucosa and aberrant crypt foci ($P < 0.01$; Student's *t*-test) (Figure 6A). Because aberrant crypt foci showed low methylation levels and all of them were regarded as LME by the established two-step panel (Figure 4C), the aberrant crypt foci could not be divided

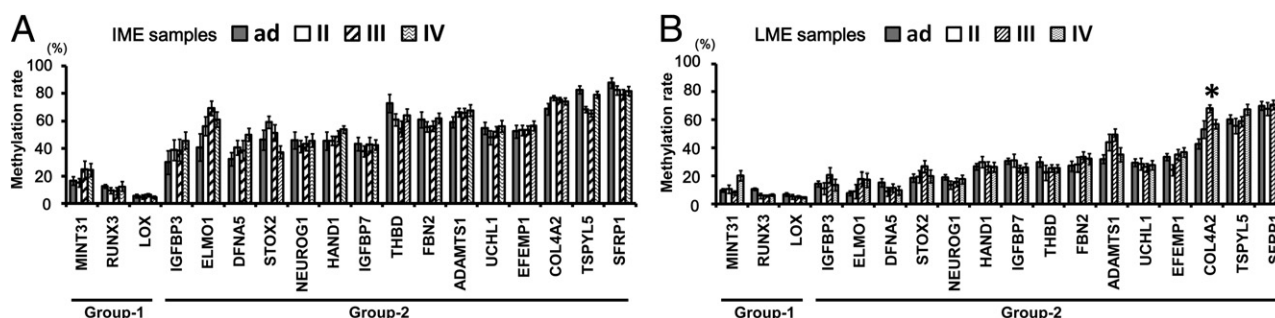


Figure 5. Comparison of average methylation rate between adenoma and cancer. Methylation levels were shown by average methylation rate \pm SE, and were compared among conventional adenoma (ad) ($n = 48$), stage II cancer (II) ($n = 35$), stage III cancer (III) ($n = 43$), and stage IV cancer (IV) ($n = 52$), separately in intermediate methylation epigenotype (IME) (A) and low methylation epigenotype (LME) (B). **A:** Comparison of IME adenoma ($n = 13$), IME stage II cancer ($n = 21$), IME stage III cancer ($n = 16$), and IME stage IV cancer ($n = 26$). All of the three group-1 markers kept low methylation levels throughout adenoma and carcinoma stages. All of the 15 group-2 markers showed high methylation rates in adenoma at similar levels to cancer. There was no significant difference among adenoma and three cancer stages in any marker (Student's *t*-test). **B:** Comparison of LME adenoma ($n = 34$), LME stage II cancer ($n = 14$), LME stage III cancer ($n = 27$) and LME stage IV cancer ($n = 26$). Methylation rates were generally lower than IME. There was no significant difference of methylation rate among adenoma and three cancer stages in all of the marker except *COL4A2*, which showed methylation increase from adenoma to cancer ($*P < 0.01$, Student's *t*-test).

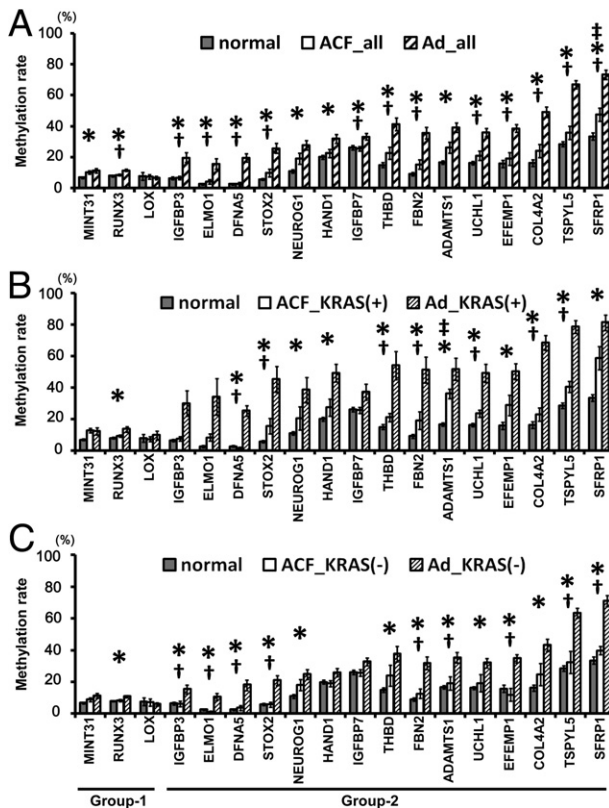


Figure 6. Comparison of average methylation rates between adenoma and aberrant crypt foci. Methylation levels were shown by average methylation rate \pm SE. **A:** Normal colorectal mucosa (n = 26), all of the aberrant crypt foci (ACF) (ACF_all, n = 13) and all of the conventional adenoma (Ad) (Ad_all, n = 48) were compared. All of the 15 group-2 markers showed significant difference between normal colorectal mucosa and adenoma (* P < 0.01, Student's *t*-test), and 12 of 15 group-2 markers between aberrant crypt foci and adenoma (* P < 0.01), whereas only SFRP1 among 15 group 2 markers showed a significant methylation difference between normal colorectal mucosa and aberrant crypt foci (* P < 0.01). **B:** Normal colorectal mucosa (n = 26), aberrant crypt foci with KRAS mutation (n = 5), and conventional adenoma with KRAS mutation (n = 11) were compared. Again, higher methylation of group-2 markers in adenoma was generally detected rather than normal mucosa or aberrant crypt foci. There were 12 of 15 group-2 markers that showed a significant difference between normal colorectal mucosa and adenoma (* P < 0.01), and 7 of 15 markers that showed a significant difference between aberrant crypt foci and adenoma (* P < 0.01), whereas only ADAMTS1 among 15 group-2 markers showed a significant methylation difference between normal colorectal mucosa and aberrant crypt foci (* P < 0.01). **C:** Normal colorectal mucosa (n = 26), aberrant crypt foci without KRAS mutation (n = 8), and conventional adenoma without KRAS mutation (n = 37) were compared. Higher methylation of group-2 markers in adenoma was detected rather than normal mucosa or aberrant crypt foci. There were 13 of 15 group-2 markers that showed a significant difference between normal colorectal mucosa and adenoma (* P < 0.01), and 9 of 15 markers showed a significant difference between aberrant crypt foci and adenoma (* P < 0.01), whereas no markers showed a significant methylation difference between normal colorectal mucosa and aberrant crypt foci.

into IME and LME, so it was classified into *KRAS*-mutation(+) and *KRAS*-mutation(-) instead (Figure 6, B and C). Also in comparison of *KRAS*-mutation(+) lesions, only *ADAMTS1* among 15 group 2 markers did show significant methylation difference between normal colorectal mucosa and aberrant crypt foci, but 12 of 15 group 2 markers showed significant difference between normal colorectal mucosa and adenoma, and 7 markers between aberrant crypt foci and adenoma (P < 0.01; Student's *t*-test) (Figure 6B). In comparison of *KRAS*-mutation(-) lesions, no markers showed significant

methylation difference between normal colorectal mucosa and aberrant crypt foci, but 13 of 15 group 2 markers showed significant difference between normal colorectal mucosa and adenoma, and 9 markers between aberrant crypt foci and adenoma (P < 0.01; Student's *t*-test) (Figure 6C).

Discussion

To gain insight into epigenotype development in colorectal carcinogenesis, especially the existence of IME and its correlation to *KRAS* mutation in colorectal adenoma, we performed a quantitative methylation analysis of group 1 and group 2 markers in 48 conventional adenomas, and compared them to 3 serrated adenomas, 149 colorectal cancers, 13 aberrant crypt foci, and 26 normal samples. The serrated adenoma showed HME and *BRAF*-mutation(+), the conventional adenoma was classified into IME and LME, and the IME showed significant correlation to *KRAS* mutation(+). Epigenotype was shown to be developed earlier than cancer progression, and it is already completed at the adenoma stage.

Whereas the conventional adenoma had been considered as the precursor lesion of colorectal cancer, hyperplastic polyps had been deemed to have no malignant potential.¹⁶ In 1990, Longacre and Fenoglio-Preiser¹⁷ analyzed a group of polyps with features similar to hyperplastic polyps and adenomas, and these unique lesions with a serrated morphology were named "serrated adenoma." It is now considered that serrated adenomas consist of traditional serrated adenoma and sessile serrated adenoma.⁹ Sessile serrated adenoma is known to share common molecular features with sporadic microsatellite-unstable colorectal cancer including *BRAF*-mutation(+) and CIMP+, and is thus considered to be a precursor of CIMP+ colorectal cancer.^{7-9,15,18} We confirmed that the one sessile serrated adenoma in this study showed HME/CIMP+ and *BRAF*-mutation(+). In less common traditional serrated adenoma,⁹ reported frequencies of *BRAF* mutation varied from low frequency as 20% (1/5 cases) compared to 75% (12/16) of sessile serrated adenoma,⁷ to high frequency as 67% (2/3) compared to 78% (28/36) of sessile serrated adenoma.¹⁵ There is also less known regarding carcinoma arising in traditional serrated adenoma.⁹ In this study, the two traditional serrated adenomas showed HME/CIMP+ and *BRAF*-mutation(+), as with the sessile serrated adenoma. Although the major purpose of this study is analysis of conventional adenomas and the sample size of serrated adenomas was as small as three, analysis of more sessile and traditional serrated adenoma samples would clarify epigenotypes and oncogene statuses within serrated adenomas.

On the other hand, any of the conventional adenoma did not show HME or *BRAF*-mutation(+). Instead, *KRAS* mutation was frequently observed (23%) in conventional adenoma. These frequencies are concordant with the previous reports,^{19,20} however, it was not been previously clarified whether there was any specific phenotype of methylation accumulation or its association to *KRAS*-mutation status in conventional adenoma. O'Brien et al²⁰

analyzed the methylation of five classic CIMP markers by methylation-specific PCR and reported that methylation level of serrated polyps is higher than conventional adenomas, which is similar to our results of group 1 marker methylation. Kim et al²¹ analyzed methylation status of seven genes also by methylation-specific PCR and distinct gene-specific methylation profile was suggested between serrated polyps and tubular adenomas. Similar to our results that IME adenomas were larger than LME adenomas, Kakar et al²² reported that higher frequency of methylation was associated with larger size of adenomas. In these previous reports, however, any methylation phenotype or its association to *KRAS* mutation within conventional adenomas had not been identified. This might perhaps be due to lack of suitable markers. Also, methylation-specific PCR is not quantitative assay, and quantitative methylation analysis is preferable for molecular classification.²³

By quantitative methylation analysis using both group 1 markers (\equiv CIMP markers) and group 2 markers that we previously developed,¹⁰ we classified conventional adenoma into IME and LME through an unsupervised hierarchical clustering method. IME adenoma correlated significantly to *KRAS*-mutation(+), as well as IME colorectal cancer.¹⁰ Moreover, there is no difference of methylation levels between IME adenoma and IME cancer. These indicated that the epigenotype development is already completed at the adenoma stage. It might also be suggested that conventional adenoma is a precursor of IME and LME colorectal cancer, and that the epigenotypes (IME or LME), which the tumor would show after cancer progression, is already determined at the adenoma stage.

Aberrant crypt foci in colorectal mucosa are the earliest known neoplastic lesions with monoclonal cell expansion.^{24–26} The cell expansions occur to a limited state, despite the frequent mutation of *KRAS*, and they showed significantly lower levels of methylation than *KRAS*-mutation(+) adenoma. Analyzed aberrant crypt foci were not numerous, although our data might suggest that methylation accumulation occurs during aberrant cell expansion in adenoma formation. Considering that oncogene mutation in normal cells could cause cellular growth arrest, the so-called oncogene-induced senescence,²⁷ perhaps methylation of group 2 marker genes in IME may be a requested aberration for further cellular growth to form *KRAS*-mutation(+) adenoma.

If methylation accumulation is completed at the adenoma stage, then there needs to be a cause for adenoma to become malignant other than the methylation of analyzed group 2 markers. One possibility is that there might be other group 2 genes that are not methylated in IME adenoma but would be methylated in IME cancer to cause malignant change. In the case of carcinogenesis for HME, such a gene can be considered to be *MLH1*; other group 1 genes are methylated, but *MLH1* is not methylated at the adenoma stage, and *MLH1* is methylated in cancer to cause microsatellite instability and mutator phenotype.²⁸ Another possibility is that genetic or genomic alterations can be the causes for adenomas to become malignant. In sporadic colorectal cancer, microsatellite instability and

chromosomal instability are well-characterized genetic alterations, and these two pathways are exclusive.²⁹ To clarify genesis of malignant formation from colorectal adenoma to cancer, further analyses should be performed, including methylation of more genes and chromosomal instability.

In this study, we found that IME and its correlation to *KRAS* mutation are developed in conventional adenoma, and that epigenotype development is already completed at adenoma stage.

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