Subsets of microsatellite-unstable colorectal cancers exhibit discordance between the CpG island methylator phenotype and *MLH1* methylation status

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Although the presence of *MLH1* methylation in microsatellite-unstable colorectal cancer generally indicates involvement of the CpG island methylator phenotype (CIMP) in the development of the tumor, these two conditions do not always correlate. A minority of microsatellite-unstable colorectal cancers exhibit discordance between CIMP and *MLH1* methylation statuses. However, the clinicopathological features of such micro-satellite-unstable colorectal cancers with discrepant *MLH1* methylation and CIMP statuses remain poorly studied. Microsatellite-unstable colorectal cancers ($n = 220$) were analyzed for CIMP and *MLH1* methylation statuses using the MethyLight assay. Based on the combinatorial CIMP and *MLH1* methylation statuses, the microsatellite-unstable colorectal cancers were grouped into four subtypes (CIMP-high (CIMP-H) *MLH1* methylation-positive (MLH1m+), CIMP-H *MLH1* methylation-negative, CIMP-low/0 (CIMP-L/0) MLH1m+, and CIMP-L/0 *MLH1* methylation-negative), which were compared in terms of their associations with clinicopatho-logical and molecular features. The CIMP-L/0 *MLH1* methylation-negative and CIMP-H MLH1m+ subtypes were predominant, comprising 63.6 and 24.1% of total microsatellite-unstable colorectal cancers, respectively. The discordant subtypes, CIMP-H *MLH1* methylation-negative and CIMP-L/0 MLH1m+, were found in 5 and 7% of microsatellite-unstable colorectal cancers, respectively. The CIMP-H *MLH1* methylation-negative subtype exhibited elevated incidence rates in male patients and was associated with larger tumor size, more frequent loss of MSH2 expression, increased frequency of *KRAS* mutation, and advanced cancer stage. The CIMP-L/0 MLH1m+ subtype was associated with onset at an earlier age, a predominance of MLH1 loss, and earlier cancer stage. None of the CIMP-L/0 MLH1m+ subtype patients succumbed to death during the follow-up. Our findings suggest that the discordant subtypes of colorectal cancers exhibit distinct clinicopathological and molecular features, although the proportion of discordant subtypes is low. The microsatellite-unstable colorectal cancers of the same CIMP status tended to exhibit different clinicopathological features depending on *MLH1* methylation status.

Colorectal cancer is the third most common cancer and the third leading cause of cancer deaths in the USA, independent of gender.[1] In Korea, the incidence of colorectal cancer has rapidly increased during the past decade (1999–2008), becoming the second and fourth most common cancer in Korean men and women, respectively (http://www.cancer.go.kr/). Colorectal cancer is a heterogeneous disease in terms of molecular carcinogenesis, and at least three molecular pathways are involved in colorectal carcinogenesis, including chromosomal instability, microsatellite instability, and the CpG island methylator pheno-type (CIMP) pathway.[2] Chromosomal instability is

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involved in the tumorigenesis and progression of the vast majority of the cancers occurring in the left large bowel (distal to the splenic flexure), whereas for cancers occurring in the right large bowel, chromosomal instability, microsatellite instability, and CIMP have important roles in tumorigenesis and tumor progression.[3,4] Defects of the mismatch repair enzymes, including *MLH1* and *MSH2*, generate microsatellite instability, which acts as the driving force for the tumorigenesis and progression observed in Lynch syndrome tumors. Apart from genetic mutations, promoter CpG island hypermethylation can inactivate *MLH1*, which is causally associated with sporadic microsatellite-unstable colorectal cancers. In contrast with the germline mutations of *MLH1* or *MSH2*, which lead to the development of microsatellite-unstable conventional adenomas,[5–7] *MLH1* promoter CpG island methylation is believed to occur during the progression of sessile serrated adenomas along the serrated neoplasia pathway.[4,8–10] Thus, *MLH1* promoter CpG island methylation is believed to act not as an initiator but as a modifier during molecular carcinogenesis. As *MLH1* methylation is generally accompanied by concordant methylation of multiple CpG island loci,[11,12] *MLH1* methylation-positive (MLH1m+) tumors tend to also be CIMP-high (CIMP-H) microsatellite-unstable tumors.

As CIMP-H microsatellite-unstable colorectal cancers harbor genes that are inactivated not only by microsatellite instability but also by CIMP, the repertoire of inactivated genes in these colorectal cancers is likely to be distinct from (and possibly more extensive than) that exhibited by the CIMP-low/0 (CIMP-L/0) microsatellite-unstable colorectal cancers observed in Lynch syndrome.[13] These differences in the repertoire of the inactivated genes between CIMP-H microsatellite-unstable colorectal cancers and CIMP-L/0 microsatellite-unstable colorectal cancers are expected to reflect the phenotypic differences between them. Jass,[14] Jass et al[15] and Young et al[16] were the first to notify and to report the differences between these cancers.[14–16] Several studies have since focused on and addressed this issue,[17–20] showing that CIMP-H microsatellite-unstable colorectal cancers exhibit increased incidence rates in female patients, increased localization to the proximal large bowel, later age of onset, more frequent poor differentiation, increased frequency of *BRAF* mutation, increased nodal metastasis, and shortened survival time.

Our previous study conducted to identify prognostic factors in microsatellite-unstable colorectal cancers found that CIMP-H status, *BRAF* mutation, poor differentiation, and advanced cancer stage were indices of poor prognosis in patients with microsatellite-unstable colorectal cancers.[17] In that study, we found that all of the microsatellite-unstable colorectal cancers with *MLH1* methylation examined were not CIMP-H, and furthermore, that all of the CIMP-H microsatellite-unstable colorectal

cancers examined were not positive for *MLH1* methylation. However, no study has reported the presence of CIMP-H microsatellite-unstable colorectal cancers without *MLH1* methylation. Therefore, the clinicopathological features of this colorectal cancer subset remain unknown. Moreover, our understanding of the clinicopathological features of CIMP-L/0 microsatellite-unstable colorectal cancers with *MLH1* methylation remains limited. In this study, we classified microsatellite-unstable tumors into four subtypes depending on the combinatorial status of CIMP and *MLH1* methylation and aimed to characterize the clinicopathological and molecular features of these four subtypes of microsatellite-unstable colorectal cancers.

Materials and methods

In all, 78 patients with microsatellite-unstable colorectal cancer(s) (7.9%) were identified from a consecutive series of 989 patients who received curative surgery for colorectal cancers at Seoul National University Hospital (Seoul, South Korea) between 2004 and 2006. Six patients were excluded from the study because of the unavailability of paraffin-tissue blocks or a history of preoperative radiation. From a consecutive series of 2478 patients with colorectal cancer(s), who underwent curative surgery in Asan Medical Center (Seoul, South Korea), between 2004 and 2006, 170 patients (7%) were found to have microsatellite-unstable colorectal cancer(s). Of these 170 cases, 148 cases were available for the molecular study. From the surgical files of the Department of Pathology, Seoul National University Hospital (Seoul, Korea) and of the Department of Diagnostic Pathology, Asan Medical Center (Seoul, Korea), we retrieved formalin-fixed, paraffin-embedded archival tissue blocks of microsatellite-unstable colorectal cancers. For patients with multiple microsatellite-unstable colorectal cancers, we analyzed individual tumors with advanced tumor staging or larger tumor size if individual tumors were of the same tumor stage. The electronic medical records were reviewed for the clinicopathological information of the 220 patients. Staging of the disease was classified according to the sixth edition of guidelines of the American Joint Committee on Cancer. This study was approved by the Institutional Review Board.

Histological Assessment

Through microscopic examination of hematoxylin- and eosin-stained tissue glass slides, all of the cases were evaluated for the following histological parameters: tumor necrosis, papillary or villous appearance, acinar-form appearance, signet ring cell appearance, serrated appearance, eosinophilic cytoplasm, vesicular nuclei, extraglandular interstitial

mucin, intraglandular dirty mucin, Crohn's-like lymphoid reaction, and intratumoral periglandular lymphoid cell infiltration. The defining criteria for these parameters were described previously.[17] Medullary carcinoma was defined as a tumor that shows sheets of tumor cells with vesicular nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm.

DNA Isolation, the Analysis of *KRAS* and *BRAF* Mutations, and the Determination of Microsatellite Instability

Under microscopic examination, the tumor areas that were most representative of the tumor histology and differentiation, as well as those that were most dense in tumor cellularity, were selected and marked. The marked areas were manually scraped with a scalpel blade and the scraped tissue samples were collected into 1.5 ml microtubes containing 30 μl tissue-lysis buffer (proteinase K (3 mg/ml) and digestion solution (50 mM Tris, 1 mM EDTA, pH 8.0, and 1% Tween-20)). The samples were incubated at 55 °C for up to 48 h. Following centrifugation, the supernatants were transferred into a newly labeled microtube. The samples were then placed into a 95 °C heat block for 10 min to inactivate the proteinase K. The mutational status of the *KRAS* codons 12 and 13 and *BRAF* codon 600 was assessed by PCR and sequence analysis as described previously.[19,21] Microsatellite instability was assessed for the five NCI reference loci, including BAT25, BAT26, D2S123, D5S346, and D17S150. A tumor was classified as microsatellite-unstable or high-level microsatellite instability when it exhibited microsatellite instability in at least two of the five microsatellite markers.

CIMP Determination

DNA samples were bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The modified DNA samples were analyzed for their methylation status of eight CIMP-panel markers (*CACNA1G*, *CDKN2A* (*P16*), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) using a methylation-specific, probe-based, real-time PCR technology (the MethyLight assay). The DNA methylation of each examined marker was quantified and reported as a percent of methylated reference (PMR). PMR $= 100 \times$ ((methylated reaction/ALU)sample/(methylated reaction/ALU)M.SssI-reference). We considered a CpG island locus methylated when an exponential amplification curve was present and the PMR value was >4. The tumors were classified as CIMP-high (CIMP-H), CIMP-L, or CIMP-0 if the tumor exhibited methylation of >4, 1–4, or no marker(s), respectively. MethyLight assay has been evaluated and validated

for its precision and performance characteristics.[22,23]

Immunohistochemistry

Sections (5 μm thick) were subjected to antigen retrieval in 10 mmol/l citrate buffer (pH 6.0) using microwave irradiation. After blocking with 3% H_2O_2 and 10% normal goat serum, the sections were incubated with either a monoclonal anti-MSH2 antibody (Oncogene Science, Cambridge, MA, USA) or a monoclonal anti-hMLH1 (PharMingen, San Diego, CA, USA) for the immunohistochemical staining of the tumor cell nuclei. Nuclear expression of these proteins was classified as present or absent relative to the staining of non-neoplastic cell nuclei, which provided a positive internal control for each slide. The immunohistochemical evaluation was performed by two pathologists (JMB and MJK) who were blinded to the DNA methylation statuses of the cases.

Statistics

The statistical analyses were performed using the statistics program SPSS software (SPSS version 15.0, Chicago, IL, USA). All of the *P*-values were two-sided, and statistical significance was set at $P < 0.05$. The χ^2 test (or Fisher's exact test for categories with an *n* value of <10) was performed on categorical data, and the two-tailed Student's *t*-test was used to evaluate the significance of the differences between two means. Disease-free survival was measured from the date of resection of the colorectal cancer to the date of recurrence, second primary colon cancer, or death from any cause, which occurred first, or to the date of the last clinical follow-up (before 31 May 2010). The disease-free survival rates were assessed with the Kaplan–Meier log-rank test. To control for confounding factors, we used the Cox proportional-hazards regression model to calculate the hazard ratio of death for each prognostic parameter found for the Kaplan–Meier log-rank tests.

Results

Subsite Distribution of Microsatellite-Unstable Colorectal Cancers

To identify whether the frequency of high-level microsatellite instability gradually changes along the large bowel from the cecum to the rectum or abruptly at the splenic flexure, we compared the frequency of high-level microsatellite instability according to bowel subsites. Tumors located in the cecum, ascending colon, transverse colon, or descending colon showed frequencies of high-level microsatellite instability $>17\%$, whereas tumors located in sigmoid or rectum displayed frequencies

of high-level microsatellite instability <7% (Figure 1). No difference of high-level microsatellite instability frequency was noted between transverse colon and descending colon, but a significant difference of high-level microsatellite instability frequency was present between descending colon and sigmoid colon (17 *vs* 6%, *P*-value <0.001, Fisher's exact test).

Four Subtypes of Microsatellite-Unstable Colorectal Cancers

We analyzed the methylation status of eight CIMP-panel markers using the MethyLight assay and determined the status of CIMP and *MLH1* methylation of 220 cases of microsatellite-unstable tumors. Of the tumors examined, 29 and 31% of the cases were positive for CIMP and *MLH1* methylation, respectively. In contrast to the general notion that CIMP-H microsatellite-unstable tumors are positive for *MLH1* methylation, 12% ($n = 27$) of the microsatellite-unstable cases exhibited discordant CIMP and *MLH1* methylation status. Of the CIMP-H tumors ($n = 64$), 17.2% ($n = 11$) were *MLH1* methylation-negative (MLH1m−), whereas of the MLH1m+ tumors ($n = 69$), 23.2% ($n = 16$) were CIMP-L/0. Based on the combinatorial statuses of CIMP and *MLH1* methylation, the microsatellite-unstable tumors could be classified into the following four subtypes: CIMP-L/0 MLH1m− ($n = 140$, 64%), CIMP-L/0 MLH1m+ ($n = 16$, 7%), CIMP-H MLH1m− ($n = 11$, 5%), and CIMP-H MLH1m+ ($n = 53$, 24%) (Figures 2 and 3). To exclude the possibility that the absence of *MLH1* methylation in the CIMP-H MLH1m− subtype or the presence of *MLH1* methylation in the CIMP-L/0 MLH1m+ subtype of colorectal cancers might be attributed to technical errors, we performed the MethyLight analyses in triplicate using two different sets of MethyLight primers and probes that targeted the proximal and distal regions of *MLH1* promoter. We found consistent results, thereby excluding the influence of technical errors in the *MLH1* Methy-Light analyses (data not shown).

The Clinicopathological Features of Four Subtypes of Microsatellite-Unstable Colorectal Cancers

The average age of the CIMP-L/0 MLH1m+ subtype patients was 53.2 years, similar to that of the CIMP-L/0 MLH1m− subtype patients (53.1 years). In contrast, the average age of the CIMP-H MLH1m− subtype patients (average, 60.0 years) was higher than that of the CIMP-L/0 MLH1m− subtype patients but lower than that of the CIMP-H MLH1m+ subtype patients (average, 66.2 years) ($P = 0.104$ and 0.052, Student's *t*-test, respectively) (Figure 4a). The male-to-female patient ratio was similar between the CIMP-L/0 MLH1m− and the CIMP-L/0 MLH1m+ subtypes, whereas the male-to-
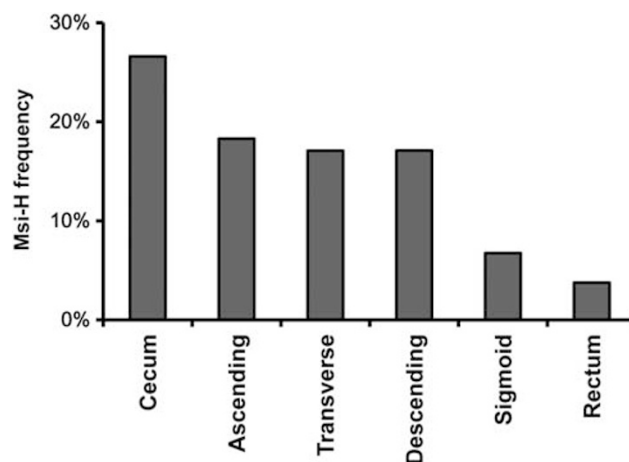

Figure 1 Frequencies of microsatellite instability-high in colorectal cancers along bowel subsites.
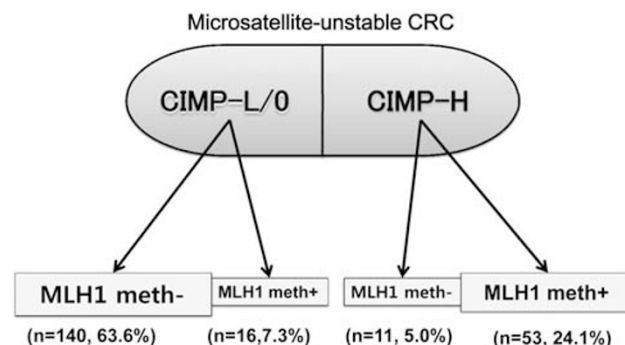

Figure 2 The four subtypes of microsatellite-unstable colorectal cancers according to the combinatorial statuses of CpG island methylator phenotype and *MLH1* methylation.

female patient ratio of the CIMP-H MLH1m− subtype was significantly different from that of the CIMP-H MLH1m+ subtype (10:1 *vs* 24:29, $P = 0.007$, Fisher's exact test, two sided) (Table 1). The preponderance of the right colon was more distinct in the CIMP-H MLH1m+ and CIMP-H MLH1m− tumor subtypes than in the CIMP-L/0 MLH1m− and CIMP-L/0 MLH1m+ tumor subtypes (85 and 73% *vs* 60 and 56%, respectively, $P = 0.009$, χ^2 test). Multiple synchronous adenocarcinomas within the large bowel were detected at a frequency of >10% in all of the subtypes except for the CIMP-H MLH1m+ subtype, which only gave rise to single cancers ($P = 0.003$, χ^2 test). The CIMP-H MLH1m+ and CIMP-H MLH1m− subtype tumors were larger than CIMP-L/0 MLH1m− and CIMP-L/0 MLH1m+ tumors (Figure 4b). Poorly differentiated adenocarcinomas were more frequent in the CIMP-H MLH1m+ and CIMP-H MLH1m− tumor subtypes (43 and 36%, respectively) than in the CIMP-L/0 MLH1m+ and CIMP-L/0 MLH1m− tumor subtypes (25 and 17%, respectively) ($P = 0.005$, χ^2 test). Nodal metastasis was more frequent in the CIMP-H MLH1m− (46%) and the CIMP-H MLH1m+ (40%) tumor subtypes than in the CIMP-L/0 MLH1m− (26%) and the CIMP-L/0 MLH1m+
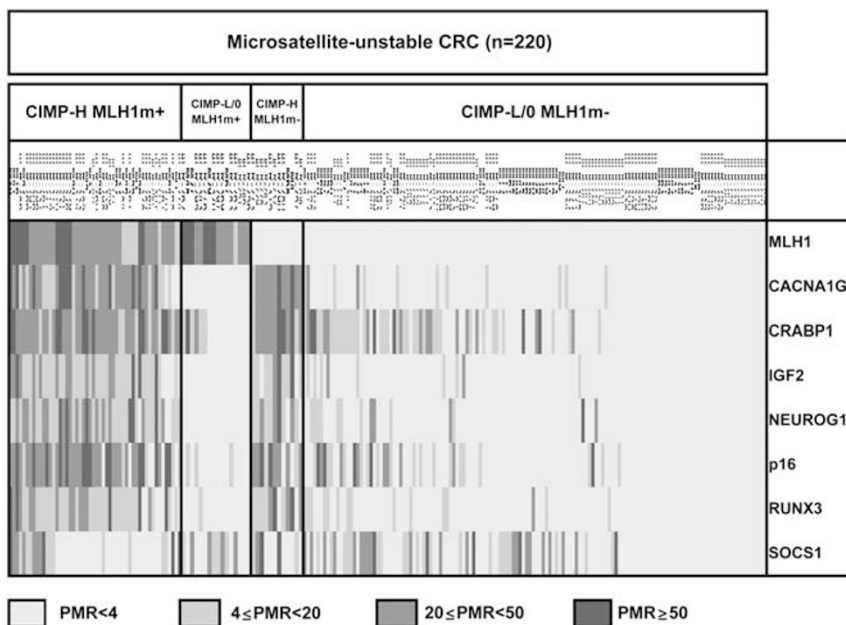
Figure 3 The methylation map of eight CpG island methylator phenotype (CIMP) markers for 220 microsatellite-unstable colorectal cancer samples.
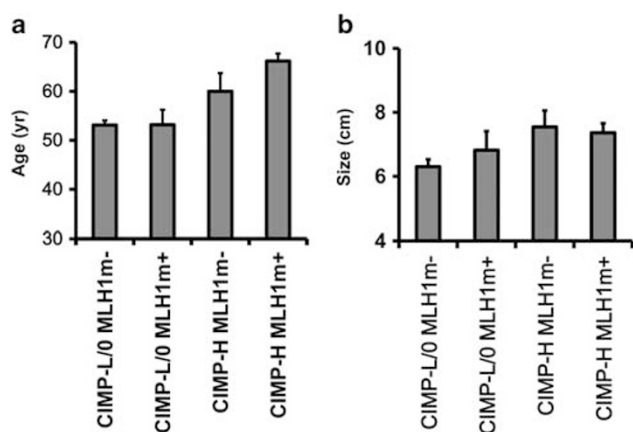

Figure 4 (**a**) The average age of the patients with each subtype of microsatellite-unstable colorectal cancer. (**b**) The average tumor size of the four subtypes of microsatellite-unstable colorectal cancer.

tumor subtypes (13%) ($P = 0.066$, χ^2 test). The CIMP-H MLH1m + tumor subtype exhibited more frequent acinar form, and signet ring cell appearances but less frequent papillary appearances and stratified nuclei compared with the other three tumor subtypes. The frequency of medullary carcinoma was not different between the four subyptes.

The Comparison of Molecular Features Between Four Colorectal Cancer Subtypes

The *BRAF* mutation was detected at a frequency of 34% in the CIMP-H MLH1m + subtype tumors and at frequencies <10% in the other three subtypes

($P < 0.001$, χ^2 test). In contrast, the *KRAS* mutation was detected in the CIMP-H MLH1m + subtype tumors at a frequency of 8%, in contrast to the other three subtypes, which exhibited *KRAS* mutation frequencies of >15%. ($P = 0.002$, χ^2 test). In particular, the *KRAS* mutation was detected in the CIMP-H MLH1m − subtype tumors at a frequency of 55%. Immunohistochemical analyses revealed that the CIMP-H MLH1m − subtype tumors exhibited loss of MLH1 (27%) and MSH2 (55%), whereas the CIMP-H MLH1m + subtype tumors exhibited predominant loss of MLH1 (85%) compared with MSH2 (9%). For the CIMP-L/0 MLH1m − subtype tumors, the loss of MLH1 and MSH2 were found in 42 and 51% of the cases, respectively. In contrast, the CIMP-L/0 MLH1m + subtype tumors exhibited loss of MLH1 and MSH2 in 94 and 6% of the cases, respectively.

Survival Analysis

Kaplan–Meier analysis of DFS revealed that TNM stage, CIMP status, Crohn's-like lymphoid reaction, and histological differentiation represent statistically significant prognostic factors in patients with microsatellite-unstable colorectal cancer (Table 2). Furthermore, age, *BRAF* mutation, and peritumoral lymphocytic reaction were marginally significant factors. Those seven factors, which were identified to be significant or marginally significant parameters by Kaplan–Meier survival analysis, were included into the multivariate analysis that was performed using the Cox proportional hazard methods model. The TNM stage and CIMP status were found to be independent prognostic parameters.

Table 1 Comparison of clinicopathological features between four molecular subtypes of microsatellite-unstable colorectal cancers

	CIMP-L/0 MLH1m− (n = 140)	CIMP-L/0 MLH1m+ (n = 16)	CIMP-H MLH1m− (n = 11)	CIMP-H MLH1m+ (n = 53)	P-value
Gender					
Male	90 (64%)	9 (56%)	10 (91%)	24 (45%)	0.016
Female	50 (36%)	7 (44%)	1 (9%)	29 (55%)	
Age (years)					
≤55	83 (59%)	9 (56%)	5 (45%)	10 (19%)	<0.001
≥56	57 (41%)	7 (44%)	6 (55%)	43 (81%)	
Location					
Right colon	84 (60%)	9 (56%)	8 (73%)	45 (85%)	0.018
Left colon	28 (20%)	5 (31%)	3 (27%)	4 (8%)	
Rectum	28 (20%)	2 (13%)	0	4 (8%)	
Gross type					
Polypoid	15 (11%)	0	0	0	0.088
Ulcerofungating	112 (80%)	14 (88%)	11 (100%)	46 (87%)	
Ulceroinfiltrative	13 (9%)	2 (13%)	0	7 (13%)	
Multiplicity					
Single	125 (89%)	12 (75%)	8 (73%)	53 (100%)	0.003
Multiple	15 (11%)	4 (25%)	3 (27%)	0	
Differentiation					
Well	15 (11%)	0	1 (9%)	1 (2%)	0.005
Moderate	101 (72%)	12 (75%)	6 (55%)	29 (55%)	
Poor	24 (17%)	4 (25%)	4 (36%)	23 (43%)	
Histological subtype					
Medullary carcinoma	7 (5%)	2 (13%)	1 (9%)	6 (11%)	0.357
Non-medullary	133 (95%)	14 (88%)	10 (91%)	47 (89%)	
T stage					
T1 and 2	28 (20%)	1 (6%)	2 (18%)	3 (6%)	0.066
T3 and 4	112 (80%)	15 (94%)	9 (82%)	50 (94%)	
N stage					
N0	104 (74%)	14 (88%)	6 (55%)	32 (60%)	0.105
N1 and 2	36 (26%)	2 (13%)	5 (45%)	21 (40%)	
M stage					
M0	135 (96%)	16 (100%)	10 (91%)	51 (96%)	0.673
M1	5 (4%)	0	1 (9%)	2 (4%)	
KRAS[a]					
Wild type	110 (81%)	10 (67%)	5 (45%)	47 (92%)	0.002
Mutant type	26 (19%)	5 (33%)	6 (55%)	7 (8%)	
BRAF[b]					
Wild type	134 (96%)	15 (94%)	10 (91%)	34 (64%)	<0.001
Mutant type	5 (4%)	1 (6%)	1 (9%)	19 (36%)	
Immunohistochemistry					
MLH1 loss	56 (42%)	15 (94%)	3 (27%)	45 (85%)	<0.001
MSH2 loss	69 (51%)	1 (6%)	6 (55%)	5 (9%)	
Retained	10 (7%)	0	2 (18%)	3 (6%)	

[a]*KRAS* sequencing analysis failed for 109 cases.
[b]*BRAF* mutant allele-specific PCR failed for one case.

When we analyzed the four tumor subtypes for their disease-free survival rates, we found that there was a significant difference in disease-free survival between the molecular subtypes: 5-year disease-free survival, 94% in the CIMP-L/0 MLH1m+ subtype, 89% in the CIMP-L/0 MLH1m− subtype, 73% in the CIMP-H MLH1m+ subtype, and 60% in the CIMP-H MLH1m− subtype ($P = 0.0067$; Figure 5). No case of the CIMP-L/0 MLH1m+ subtype succumbed to death but one case developed tumor recurrence in the lung.

Table 2 Univariate and multivariate analysis of disease-free survival in microsatellite-unstable colorectal carcinomas ($n = 220$)

Parameters	n	Univariate analysis		Multivariate analysis	
		Mean survival time (months; 95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
CIMP			0.0007		0.025
Negative	156	86 (82–90)		Reference	
Positive	64	77 (67–87)		2.249 (1.105–4.574)	
Age (years)			0.0573		0.966
55 or less	107	86 (81–90)		—	
56 or more	113	85 (78–92)		1.020 (0.413–2.519)	
BRAF			0.0781		0.424
Wild	193	83 (79–87)		—	
Mutant	26	77 (61–94)		1.465 (0.575–3.737)	
AJCC staging			<0.0001		<0.001
Stage I and II	153	89 (86–92)		Reference	
Stage III	59	76 (66–87)		4.810 (2.117–10.931)	
Stage IV	8	25 (7–42)		23.855 (8.653–65.764)	
Differentiation			0.0055		0.080
Differentiated	165	92 (88–97)		—	
Poorly differentiated	55	72 (62–82)		1.899 (0.927–3.892)	
Crohn's-like lymphoid reaction			0.0014		0.171
Absent	28	66 (51–80)		—	
Mild or marked	191	91 (87–95)		0.561 (0.245–1.283)	
Peritumoral lymphocytic reaction			0.0645		0.289
Absent or mild	168	83 (78–88)		—	
Marked	46	87 (82–92)		0.518 (0.154–1.746)	
Chemotherapy			0.7297		
Not treated	123	83 (77–89)			
Treated	95	88 (82–94)			

Abbreviations: MSI, microsatellite instability; CIMP, CpG island methylator phenotype; AJCC, American Joint Committee on Cancer.
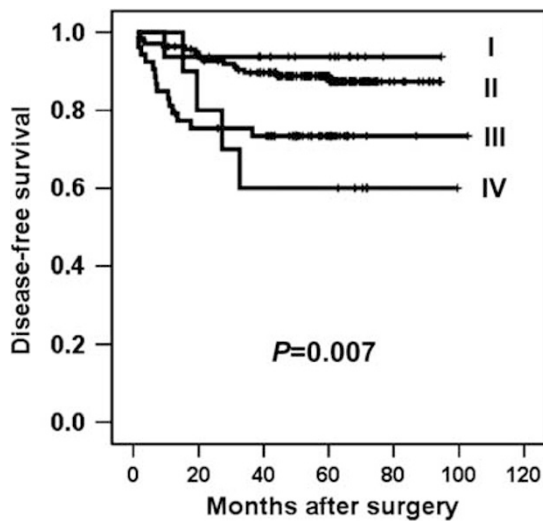

Figure 5 Kaplan–Meier curves of disease-free survival. The Kaplan–Meier survival analysis with log-rank test was performed for patients representing the following four subtypes of microsatellite-unstable colorectal cancers: CpG island methylator phenotype-low (CIMP-L)/0 *MLH1* methylation-positive (MLH1m+) (I, $n = 16$), CIMP-L/0 *MLH1* methylation-negative (MLH1m−) (II, $n = 140$), CIMP-H MLH1m+ (III, $n = 53$), and CIMP-H MLH1m− (IV, $n = 11$).

Discussion

Our data clearly demonstrate the presence of microsatellite-unstable cases with discordant CIMP and *MLH1* methylation status. We found that 17% of CIMP-H microsatellite-unstable tumors were MLH1m−, and 10% of CIMP-L/0 microsatellite-unstable tumors were MLH1m+. We excluded the possibility that the discrepancy between CIMP status and *MLH1* methylation status might be attributed to the technical errors of the MethyLight assay by performing the assay in triplicate using two different sets of MethyLight primers and probe that targeted the proximal and distal regions of *MLH1* promoter. To exclude the possibility that the loss of the MSH2 protein in the CIMP-H MLH1m− subtype tumors might be caused by *MSH2* promoter CpG island hypermethylation, we analyzed these tumors for methylation of *MSH2* using the MethyLight assay, which revealed that none of the cases harbored *MSH2* promoter CpG island hypermethylation (data not shown). This finding indicates that the aberrant hypermethylation induced by CIMP does not involve the *MSH2* promoter CpG island locus.

In our study, MLH1m+ microsatellite-unstable tumors were heterogeneous in terms of clinicopathological and molecular features that correlated with CIMP status. Compared with the CIMP-H MLH1m+ tumors, the CIMP-L/0 MLH1m+ subtype tumors were associated with an earlier age of onset (53.2 *vs* 66.2 years, $P < 0.001$, Student's *t*-test), reduced frequency of *BRAF* mutation (6 *vs* 34%, $P = 0.027$, two-sided Fisher's exact test), a preponderance toward synchronous multiplicity (25 *vs* 0%, $P = 0.002$, two-sided Fisher's exact test), reduced predilection toward right colon (56 *vs* 85%, $P = 0.033$, two-sided Fisher's exact test), reduced frequency of nodal metastasis (13 *vs* 40%, $P = 0.068$, two-sided Fisher's exact test), reduced frequency of poor differentiation (25 *vs* 43%, $P = 0.248$, two-sided Fisher's exact test), and improved prognosis. Of the eight CIMP-panel markers, *CACNA1G*, *IGF2*, and *NEUROG1* were not methylated in the CIMP-L/0 MLH1m+ subtype tumors, in contrast to the increased methylation frequencies ($>90\%$) observed in the CIMP-H MLH1m+ subtype tumors (Figure 3). These findings suggested that the CIMP-L/0 MLH1m+ and the CIMP-H MLH1m+ subtype tumors are distinct entities and that the presence of *MLH1* methylation does not necessarily correlate with the involvement of CIMP development of these microsatellite-unstable tumors.

The CIMP-L/0 MLH1m+ subtype tumors were similar to those of the CIMP-L/0 MLH1m− subtype in several regards. Features shared in common include earlier age of onset, reduced predilection toward the anatomical subsite distribution to the right colon, and multiplicity within the large bowel. However, the CIMP-L/0 MLH1m+ subtype was distinct from the CIMP-L/0 MLH1m− subtype in that it exhibited significantly different frequencies of loss of *MSH2* and *MLH1* expression. Furthermore, the patients of the CIMP-L/0 MLH1m+ subtype did not succumb to death during the follow-up period after operation. Considering three features of the CIMP-L/0 MLH1m+ subtype—the earlier age of onset, reduced number of methylated CIMP-panel markers ($n = 1.125$, except for *MLH1* methylation), and rare *BRAF* mutation rate—it is unlikely that this subtype arises from pre-existing serrated adenomas. Several studies have documented frequencies of *MLH1* methylation ranging from 0 to 55% in Lynch syndrome tumors,[24–29] suggesting that an epigenetic mechanism might act as the second hit required to inactivate the wild-type allele in Lynch syndrome tumors. In Lynch syndrome tumors exhibiting *MLH1* methylation, the number of methylated CIMP-panel markers has been demonstrated to be significantly lower than that of sporadic microsatellite-unstable tumors,[11,25] indicating that the *MLH1* methylation observed in the sporadic setting is a reflection of a widespread process of CIMP, whereas the *MLH1* methylation in Lynch syndrome tumors is a more directed event.

In this study, we first reported the incidence of the CIMP-H MLH1m− tumor subtype at 5% in micro-satellite-unstable colorectal cancers. This subtype can be seen in Wong *et al*'s study (2 of 141 microsatellite-unstable colorectal cancers), although the presence of this subtype did not gain an attention.[30] This subtype is characterized by increased incidence in male patients and a high frequency of loss of MSH2 expression. The average age of the patients with this subtype tended to be older than patients with the CIMP-L/0 MLH1m+ or CIMP-L/0 MLH1m− subtype tumors. The CIMP-H MLH1m− subtype tumors are distinct from the CIMP-H MLH1m+ subtype tumors in the following regards: (1) the male-to-female ratio was significantly different between the two subtypes—the CIMP-H MLH1m− subtype occurred predominantly in the male patients, whereas the CIMP-H MLH1m+ subtype occurred predominantly in the female patients; (2) the *KRAS/BRAF* mutation rates were significantly different between these two subtypes—the *BRAF* mutation was predominantly detected in the CIMP-H MLH1m+ subtype tumors, whereas the *KRAS* mutation was predominantly detected in the CIMP-H MLH1m− subtype tumors; and (3) the loss of *MLH1* and *MSH2* expression was different between these two subtypes—the loss of *MLH1* expression was predominantly observed in the CIMP-H MLH1m+ subtype tumors, whereas the loss of MSH2 was predominantly detected in the CIMP-H MLH1m− subtype tumors. To rule out the possibility that the loss of MSH2 expression in the CIMP-H MLH1m− subtype might be caused by *MSH2* promoter CpG island hypermethylation, we analyzed the CIMP-H MLH1m− subtype tumors for methylation of *MSH2* using the MethyLight assay, which revealed that none of the cases harbored *MSH2* promoter CpG island hypermethylation (data not shown). Some of the clinicopathological features of the CIMP-H MLH1m− subtype tumors appeared similar to those of the CIMP-L/0 MLH1m− subtype tumors, including the increased frequency of loss of MSH2 expression, increased incidence in male patients, multiplicity, and rare *BRAF* mutation rates. Considering that the CIMP-H MLH1m− subtype was associated with loss of MSH2 expression and rare *BRAF* mutations, we could not exclude the possibility that this subtype might represent Lynch syndrome tumors that acquired CIMP.

This study has limitations in that the information regarding the familial history of cases was not provided. Because this is a retrospective study, we could not obtain family histories from a considerable proportion of the cases. Furthermore, some family histories were so incomplete that we could not determine whether the respective cases satisfied the Amsterdam criteria II. Lastly, the genetic analysis of germline mutations of the DNA mismatch repair genes could not be performed because blood samples or fresh tissue samples were not procured from the patients.

It is well known that molecular features differ between right- and left-sided colon cancers.

However, little information was available regarding whether there is a gradual change of molecular features along the long axis from the cecum to the rectum or an abrupt change of molecular features at the splenic flexure before the publication of Yamauchi *et al*'s study results. Recently, Yamauchi *et al*.[31] analyzed molecular features of colorectal cancers for their subsite distribution in the large bowel (cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid, rectosigmoid junction, and rectum) and found gradual decreases of CIMP-H and high-level microsatellite instability frequencies from the ascending colon to the rectum, but no abrupt change at the splenic flexure. However, our study demonstrated an abrupt decrease of high-level microsatellite instability frequency from the descending colon to the sigmoid. Discrepancy between findings of Yamauchi *et al*'s study and those of our study might be attributed to the difference in the way of subsite division: we divided the large bowel into six subsites rather than nine subsites of Yamauchi *et al*'s study and the descending colon in our study might include part of the splenic flexure, which might increase MSI-high frequency of the descending colon in our study. In contrast with Yamauchi *et al*'s study in which cecal cancers showed lower frequency of MSI-high than that of ascending colon cancers, in our study, cecal cancers displayed higher frequency of MSI-high than that of ascending colon cancers.

In our study, colorectal cancer cases were bimodally distributed in terms of the number of methylated CIMP-panel markers (Supplementary Figure), but the two peaks were not separated as clearly as in Ogino *et al*'s study.[32] To explore the reason for the absence of clear separation of the two peaks, we performed the MethyLight assays of the eight markers in an independent set of colorectal cancer cases ($n = 308$) and determined the sensitivity and specificity of each CIMP-panel markers in the diagnosis of CIMP-H. All the eight markers showed high specificity ($>94\%$) for diagnosis of CIMP-H, but the sensitivity varied from 100 to 26% (Supplementary Table). When compared with the results of Ogino *et al*'s study,[32] our study showed higher specificities but lower sensitivities of individual markers, which might be related to the absence of clear separation of the two peaks.

A recent study that performed comprehensive genome-scale DNA methylation profiling of colorectal cancers has demonstrated that the MethyLight-based CIMP marker panel did not detect 21% of CIMP-H tumors identified by the Infinium DNA methylation data.[33] However, all the CIMP-H tumors identified by the MethyLight-based marker panel belonged to CIMP-H cluster based on the Infinium DNA methylation data. The misclassification error of the MethyLight-based CIMP marker panel in detecting CIMP-H might affect the results of our study. Some of the CIMP-L/0 cases in our study might be CIMP-H based on the Infinium DNA methylation data. However, in our study, the CIMP-L/0 MLH1m + subtype showed rare *BRAF* mutation and younger age of onset, which are inconsistent with the features of CIMP-H colorectal cancers.

In conclusion, microsatellite-unstable colorectal cancers can be classified into four subsets depending on the combinatorial statuses of CIMP and *MLH1* methylation, including CIMP-L/0 MLH1m − , CIMP-H MLH1m + , CIMP-L/0 MLH1m + , and CIMP-H MLH1m − (in a descending order of frequency). Our study clearly demonstrates that CIMP + status and MLH1m + status do not completely overlap and that the discordant subtypes CIMP-H MLH1m − and CIMP-L/0 MLH1m + exhibit distinct clinicopathological and molecular features. These findings suggest that CIMP status, rather than *MLH1* methylation status, is more likely to predict the clinicopathological features of microsatellite-unstable colorectal cancers.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)