Efficient molecular screening of Lynch syndrome by specific 3' promoter methylation of the *MLH1* or *BRAF* mutation in colorectal cancer with high-frequency microsatellite instability

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Abstract. It is sometimes difficult to diagnose Lynch syndrome by the simple but strict clinical criteria, or even by the definitive genetic testing for causative germline mutation of mismatch repair genes. Thus, some practical and efficient screening strategy to select highly possible Lynch syndrome patients is exceedingly desirable. We performed a comprehensive study to evaluate the methylation status of whole *MLH1* promoter region by direct bisulfite sequencing of the entire *MLH1* promoter regions on Lynch and non-Lynch colorectal cancers (CRCs). Then, we established a convenient assay to detect methylation in key CpG islands responsible for the silencing of *MLH1* expression. We studied the methylation status of *MLH1* as well as the CpG island methylator phenotype (CIMP) and immunohistochemical analysis of mismatch repair proteins on 16 cases of Lynch CRC and 19 cases of sporadic CRCs with high-frequency microsatellite instability (MSI-H). Sensitivity to detect Lynch syndrome by *MLH1* (CCAAT) methylation was 88% and the specificity was 84%. Positive likelihood ratio (PLR) was 5.5 and negative likelihood ratio (NLR) was 0.15. Sensitivity by mutational analysis of *BRAF* was 100%, specificity was 84%, PLR was 6.3 and NLR was zero. By CIMP analysis; sensitivity was 88%, specificity was 79%, PLR was 4.2, and NLR was 0.16. *BRAF* mutation or *MLH1* methylation analysis combined with MSI testing could be a good alternative to screen Lynch syndrome patients in a cost effective manner. Although the assay for CIMP status also showed acceptable sensitivity and specificity, it may not be practical because of its rather complicated assay.

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Abbreviations: CRC, colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MNR, mononucleotide repeat; CIMP, CpG island methylator phenotype; IHC, immunohistochemistry

Key words: Lynch syndrome, *BRAF*, *MLH1*, methylation, microsatellite instability

Introduction

A subset of colorectal cancer (CRC), either hereditary or sporadic, is caused by deficient mismatch repair (MMR) machinery and exhibits distinct clinicopathological characteristics compared with the majority of sporadic CRCs. The hereditary type of MMR-deficient cancer is known as Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC). However, accurate diagnosis of Lynch syndrome from sporadic CRC is sometimes difficult because of its less obvious clinicopathological characteristics compared with familial adenomatous polyposis (FAP), another type of hereditary CRC syndrome which has observable numbers of polyps in the colon and rectum. Also, germline mutation of cancer-causing MMR genes cannot always be detected in Lynch syndrome. Deficiency in MMR machinery, either by hereditary or sporadic reasons, causes instability (length alteration by slippage) of microsatellite sequences. High-level microsatellite instability (MSI-H), which is frequently observed in mononucleotide repeat markers such as *BAT25*, *BAT26*, *BAT40* (1,2), is demonstrated in a majority of MMR-deficient cancers (3-6). Accordingly, the MSI test is an ideal strategy to select highly probable Lynch syndrome patients from sporadic CRC patients. The main difference between sporadic and hereditary CRC with MMR defect is understood as follows: germline inactivation of MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) underlies HNPCC whereas

epigenetic silencing of the *MLH1* gene causes sporadic CRCs with an MMR defect (7-10).

Immunohistochemical (IHC) analysis of MMR gene products such as MSH2, MLH1, PMS2 and MSH6 in tumor samples has been applied to help in diagnosing Lynch syndrome (11). Recently, the CRCs with an MMR deficiency can simply be distinguished by a histopathological observation without IHC, because typical histopathological features of these tumors have been elucidated and been generally recognized. This will also help to diagnose Lynch syndrome.

CRCs with an MMR deficiency, including both hereditary and sporadic forms, can be diagnosed either by the MSI test or IHC study of MMR proteins. However, in order to diagnose Lynch syndrome, we need some additional approaches, such as a molecular method, to distinguish sporadic from hereditary forms of CRC with both having the MMR deficiency. In this study, we focused on the methylation patterns of the *MLH1* promoter as well as *BRAF* mutational status and CpG island methylator phenotype (CIMP) status to assess these molecular markers to help in pre-selection of Lynch syndrome at a stage between the initial MSI test and the final germline mutational analysis.

Materials and methods

Tissue samples. Tumor tissues and samples of the corresponding normal mucosa were obtained from CRC patients who had undergone curative surgery at Okayama University and the Royal Brisbane Hospitals in Brisbane, Queensland. Ethical approval was obtained and we obtained informed consent in writing from all subjects. Tumor staging was based on Dukes classification. Samples of both tumor and normal mucosal tissue were stored at -80˚C and DNA was extracted by the standard procedure involving digestion with proteinase K and phenol-chloroform extraction.

Direct sequencing of nested MSP products from the MLH1 promoter. Bisulfite-treated genomic DNA samples were amplified with primers specific for the promoter region of the *MLH1* gene. Primer sequences for the first PCR were: MLH1-F (5'-GGAGTGAAGGAGGTTAIGGGTAAGT-3') and MLH1-R (5'-ATTCACCACTATCTCITCCAACC-3'), and primer sequences for 'methylated' and 'unmethylated' for the nested PCR were: (a) M-F (5'-GTAGATGTTTTATTAG GGTCGC-3') and MLH1-R and (b) U-F (5'-GTAGATGTTT TATTAGGGTTGTGT-3') and MLH1-R, generating fragment lengths of 722 and 511 bp, respectively. Conditions of the first PCR and the nested PCR were as follows: 95˚C for 15 min; 45 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec; and finally, 7 min at 72˚C. The nested PCR products were purified using a QIAquick PCR purification kit (Qiagen) and directly sequenced using a Thermo Sequenase sequencing kit (Amersham, Corp., Piscataway, NJ) and a SQ-5500E Hitachi Automated DNA sequencer.

Bisulfite modification and detection of methylation status of multiple loci. Sodium bisulfite modification was performed using a CpGenome DNA modification kit (Intergen Co., New York, NY). The methylation status of *MINT1*, *MINT2*, *MINT31*, *CACNA1G*, $p16^{INK4a}$, $p14^{ARF}$, *COX2*, *DAPK*, *DCC* and *MGMT* was evaluated and determined by combined bisulfite restriction analysis (COBRA) using previously described procedures (12-17).

Methylation analysis for MLH1. The methylation status of *MLH1* was determined by a newly established method which we described elsewhere (18). The methylation of the CpG islands located upstream of the *MLH1* promoter was not shown to cause silencing but rather was age related. However, methylation at a 3'-small region closer to the transcription start site invariably correlated with the absence of *MLH1* expression *in vitro* and in colorectal tumors (19). Accordingly, we needed to use the two sets of primers to evaluate the methylation status of both the upstream and downstream regions. Our method enabled us to distinguish *MLH1-5'* region sequences that were methylated or unmethylated by determining whether the fragments produced were 120 or 186 bp long. A similar approach was used for PCR analysis of the *MLH1-3'* region and the methylated and unmethylated 3' region (termed CCAAT region) sequences were distinguished by the presence of, 122 and 232 bp fragments, respectively.

Microsatellite analysis. The MSI testing for each tumor was determined on the basis of an examination of 5 microsatellite markers (*BAT25*, *BAT26*, *D2S123*, *D5S346*, *D17S250*) by our previously described method (20). We classified tumors as MSI-H if two of the markers displayed MSI. Tumors displaying no MSI with any of the microsatellite markers that we tested were classified as MSS.

Detection of BRAF codon 600, KRAS codon 12 and 13 mutations. *BRAF* mutations at codon 600 and *KRAS* mutations at codons 12 and 13 were determined using standard protocols as we described previously (18). We confirmed the nature of each of the mutations that we detected by our RFLP analysis by sequencing the appropriate DNA samples in a Hitachi Autosequencer SQ-5500E used in accordance with the manufacturer's instructions.

Immunohistochemical analysis for MLH1. Immunohistochemical (IHC) staining for MLH1 (Clone G168-728, 1 mg/ml; PharMingen, San Diego, CA), MSH2 (Clone FE11, 0.5 mg/ml; Oncogene Science, Cambridge, MA), MSH6 (Clone 44, 0.5 mg/ml; Transduction Laboratory, Lexington, KY), and PMS2 (Clone A16-4; PharMingen) were performed as described previously (21). We used diamino-benzidine as a chromogen and hematoxylin as a nuclear counterstain. The only foci that were scored as negative were those for which there was definite evidence of positively staining admixed (or surrounding) non-neoplastic tissues such as normal colonic mucosal cells, lymphocytes or stromal cells. The normal staining pattern for MLH1, MSH2 and MSH6 was nuclear. Tumor cells that exhibited an absence of nuclear staining, in the presence of non-neoplastic cells with nuclear staining, were considered to have an abnormal pattern.

Statistical analysis. All statistical analysis was performed using JMP4.05J (SAS Institute, Inc., Cary, NC). Differences in frequencies were evaluated by the Fisher's exact test or Pearson's Chi-square test where appropriate. The association

among the average number of methylated loci of 3 CRC subsets was analyzed using the Wilcoxon/Kruskal-Wallis test. All reported P-values are 2-sided and a $P<0.05$ was considered statistically significant. Confidence intervals for sensitivity and specificity were based on confidence intervals for the corresponding false negative and false positive error rates considered as independent binomial random variables, using tables appropriate for small samples (22). The positive likelihood ratio (PLR) is equal to $(1-ß)/\alpha$ and the negative likelihood ratio (NLR) is equal to $ß/(1-\alpha)$, where α and ß are the false positive and false negative error rates, respectively. Confidence intervals for the positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were obtained by numerical approximation using a discretized likelihood; the nominal coverage of 95% was confirmed on all possible combinations of α and ß on the interval: $0< \alpha,ß <0.3$.

Results

Direct sequencing of bisulfite-PCR products in the MLH1 promoter region

Methylation allele-specific direct sequencing. We sequenced bisulfite-treated nested PCR products to obtain reliable information about the methylation status of the CpG islands that are known to be located in the nt 1031-1600 region of the *MLH1* promoter. Fig. 1a shows the results that we obtained in a pilot direct sequencing study of 2 MSI-H CRCs and 2 CRCs

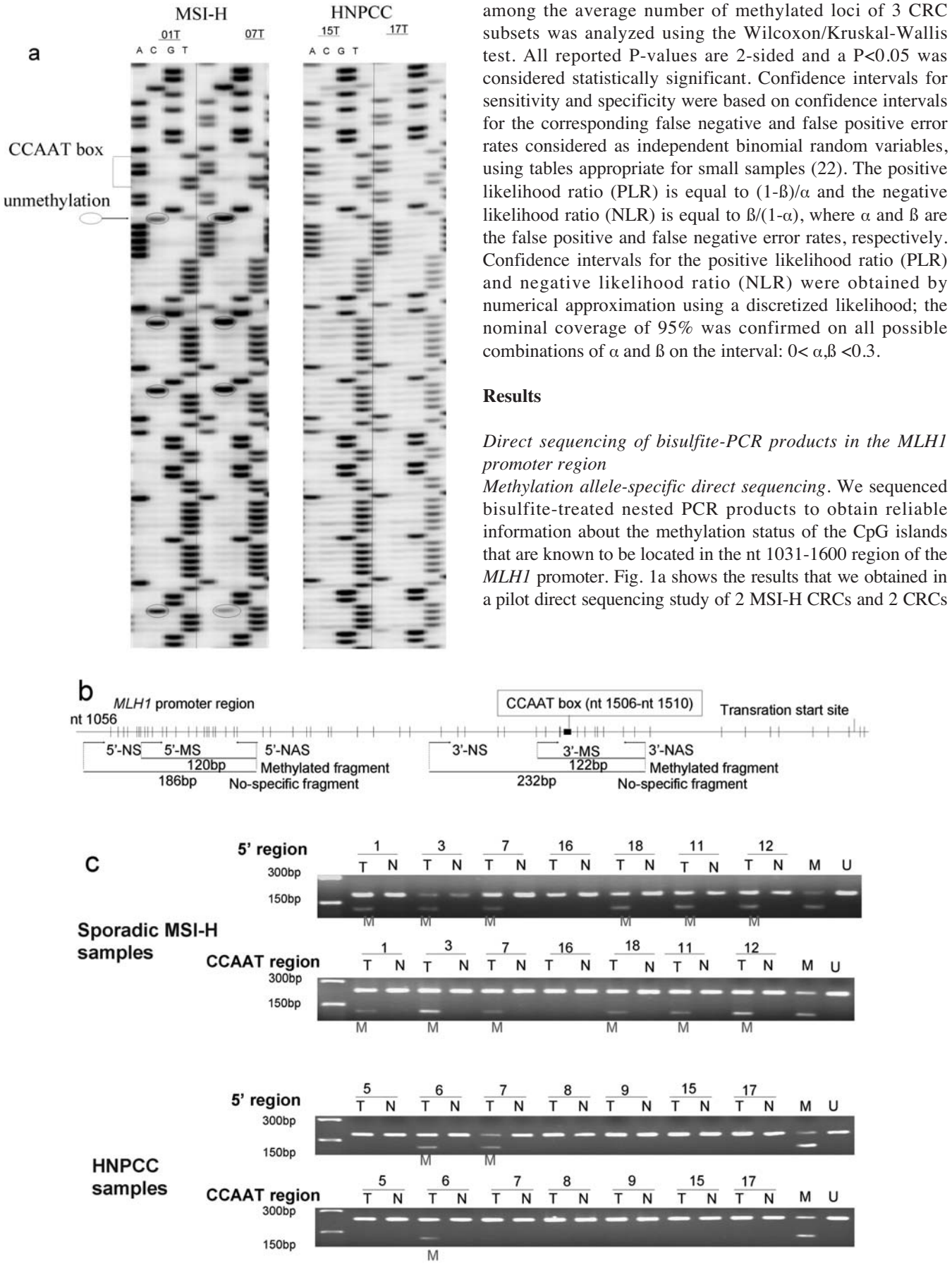

Figure 1. (a) Bisulfite sequencing of the DNA derived from colorectal cancer tissue with MSI-H and the colorectal cancer from Lynch syndrome patients. The circle indicates the unmethylated cytosine not substituted by the thymine. (b) Schematic representation of the promoter region and exon 1 of *MLH1*. The vertical lines represent individual CpG sites. CCAAT box, which specifically bound transcription factor CBF, are shown in this map. Primers of the methylation-specific single polymerase chain reaction (MSSP) are shown. (c) MSSP results of the 5' region of the *MLH1* and 3' (CCAAT box) region in sporadic colorectal cancer with MSI-H and Lynch syndrome colorectal cancer are shown.

	No.		Sex	Age	Location	Histlogy	MLH1 methylation		MLH1 IHC	Mutation analysis	IHC of MMR
							5'region	CCAAT			
Sporadic MSI-H	1	MSI-H	M	79	P	por	M	M	n		
	2	MSI-H	F	72	D	por	M	M	n		
	3	MSI-H	F	62	P	mod	M	M	n		
	4	MSI-H	F	76	P	mod	M	M	n		
	5	MSI-H	F	67	P	mod	M	M	n		
	6	MSI-H	F	79	P	por	M	M	n		
	7	MSI-H	M	72	P	por	M	M	n		
	8	MSI-H	F	74	P	mod	M	M	n		
	9	MSI-H	F	77	P	-	M	M	n		
	10	MSI-H	F	87	P	por	M	M	n		
	11	MSI-H	F	73	P	mod	M	M	n		
	12	MSI-H	M	87	P	por	M	M	n		
	13	MSI-H	F	53	P	por	M	M	n		
	14	MSI-H	M	69	P	por	M	M	n		
	15	MSI-H	F	42	P	por	M	M	n		
	16	MSI-H	F	79	P	por	U	U	n		
	17	MSI-H	M	74	D	por	M	U	n		
	18	MSI-H	F	76	P	por	M	M	n		
	19	MSI-H	M	65	D	mod	M	U	p		
Lynch syndrome (MSI-H)	1	HNPCC	M	40	P	por	U	U	n	no mutation	Absent of PMS2
	2	HNPCC	M	38	D	mod	U	U	p	no mutation	Absent of MSH2
	5	HNPCC	F	65	P	mod	U	U	p	exon5 of MSH6	Absent of MSH6
	6	HNPCC	M	43	D	mod	M	M	n	no mutation	
	7	HNPCC	M	50	P	por	M	U	p	exon1 of MSH2	Absent of MSH2
	8	HNPCC	F	31	D	mod	U	U	p	no mutation	Absent of MSH2
	9	HNPCC	F	34	D	mod	U	U	p	no mutation	Absent of MSH2
	10	HNPCC	M	27	P	por	U	U	p	whole gene deletion of MSH2	Absent of MSH2
	13	HNPCC	F	25	P	por	U	U	p	no mutation	Absent of PMS2
	14	HNPCC	F	40	P	por	U	U	n	no mutation	
	15	HNPCC	M	53	P	mod	U	U	n	exon16 of MLH1	
	16	HNPCC	M	33	P	por	U	U	n	no mutation	
	17	HNPCC	M	54	D	por	U	U	n	exon17 of MLH1	
	20	HNPCC	F	36	P	mod	M	M	n		
	22	HNPCC	M	56	D	mod	U	U	p		Absent of MSH6
	23	HNPCC	M	37	P	mod	U	U	p		

Figure 2. *MLH1* methylation status and immunohistochemical study in sporadic colorectal cancers with MSI-H and Lynch syndrome colorectal cancers met the Amsterdam Criteria.

with Lynch syndrome. The primers that we chose made it easy to distinguish between sequences containing methylated and unmethylated versions of the CpG doublets in the regions that we were targeting. Cytosine of the CpG islands in the promoter region of *MLH1* remained as cytosine in the two sporadic MSI-H CRC tissues (01T and 07T), but converted to thymine in the two CRC tissues of Lynch syndrome patients (15T and 17T).

The methylation status of the two distinct MLH1 promoter regions in CRCs of Lynch syndrome and sporadic MSI-H CRCs. We assessed the methylation status of both 5' region and 3' regions of the *MLH1* promoter using a methylation-specific single PCR (MSSP) comparing CRCs of Lynch syndrome and sporadic MSI-H CRCs (Fig. 1b). By this method we have already shown that methylation in the 3' region is highly correlated with the silencing of the *MLH1*; ie, loss of expression of MLH1 (18). In this study, methylation of the 5' region of the *MLH1* promoter was observed in 18 out of 19 sporadic MSI-H (Figs. 1b and 2), while methylation of the 3' region was observed in 16 out of 19 sporadic MSI-H. In contrast, few cases of methylation were observed in CRCs of Lynch syndrome patients: methylation of the 3' region was only detected in 2 out of 16 tumors (T6 and T20), while 5' methylation was observed in 3 out of 16 tumor samples (T6, T7 and T20) (Figs. 1b and 2).

Methylation status of MLH1 and IHC in sporadic and hereditary MMR defect colorectal cancers. Methylation status of *MLH1* and IHC results on MMR proteins are shown in Fig. 2. The majority of the sporadic MSI-H CRCs lost the expression of MLH1. However, in Lynch syndrome CRCs only 7 out of 16 samples showed loss of expression of MLH1, and germline mutation of *MLH1* was confirmed in 2 of them. Two samples showed methylation in the 3' region of *MLH1*. Five samples showed loss of expression of MSH2 and among them two samples were confirmed as germline mutations of *MSH2*. Two samples showed loss of expression of MSH6 with one germline mutation confirmed and two samples showed loss of expression of PMS2 without detection of germline mutations. One Lynch syndrome sample showed loss of expression of both MLH1 and PMS2.

Methylation status of CIMP genes and non-CIMP genes in MMR deficient colorectal cancers. Fig. 3 shows the methylation status of CIMP markers, including *MINT1*, *MINT2*, *MINT31*, $p16^{INK4a}$ and *CACNA1G*, in sporadic MSI-H CRCs and Lynch syndrome CRCs. We also assessed the methylation status of non-CIMP genes, including $p14^{ARF}$, *DAPK*, *COX2*, *MGMT* and *DCC*, for comparison. We define CIMP (+) as >3 markers showing methylation among the CIMP 5 markers. Fifteen out of 19 sporadic MSI-H cancers were determined to be CIMP (+), whereas there were no

	No.		Sex	Age	MLH1 methylation		CIMP methylation						other gene met.				*KRAS*	*BRAF*
					5'region	CCAAT	MINT2	p16	MINT31	CACNA1G	MINT1	p14	DAPK	COX2	DCC	MGMT		
Sporadic MSI-H	1	MSI-H	M	79	M	M	M	M	M	M	M	U	U	U	M	M	W	V600E
	2	MSI-H	F	72	M	M	M	M	M	M	M	U	U	U	M	U	W	V600E
	3	MSI-H	F	62	M	M	M	M	M	M	M	M	U	U	M	M	W	V600E
	4	MSI-H	F	76	M	M	M	M	M	M	M	M	U	U	M	M	W	V600E
	5	MSI-H	F	67	M	M	M	M	M	M	M	M	U	U	M	U	W	V600E
	6	MSI-H	F	79	M	M	M	M	M	M	M	M	U	U	M	M	W	V600E
	7	MSI-H	M	72	M	M	M	M	M	M	M	M	M	M	M	U	W	V600E
	8	MSI-H	F	74	M	M	M	M	M	M	M	M	U	U	M	M	W	V600E
	9	MSI-H	F	77	M	M	M	M	M	M	M	U	U	U	M	M	W	V600E
	10	MSI-H	F	87	M	M	M	M	M	M	U	U	U	U	M	M	W	V600E
	11	MSI-H	F	73	M	M	M	U	U	U	M	U	U	U	U	U	W	V600E
	12	MSI-H	M	87	M	M	M	M	M	M	M	M	M	U	M	M	W	V600E
	13	MSI-H	F	53	M	M	U	M	M	M	M	M	U	U	U	M	W	V600E
	14	MSI-H	M	69	M	M	M	M	M	M	M	M	U	U	M	U	W	V600E
	15	MSI-H	F	42	M	M	M	M	U	M	M	U	M	M	M	U	W	V600E
	16	MSI-H	F	79	U	U	U	M	U	M	U	U	U	U	M	U	W	W
	17	MSI-H	M	74	M	U	U	U	M	U	U	U	U	U	M	U	W	W
	18	MSI-H	F	76	M	M	M	M	M	M	M	M	U	M	M	U	W	V600E
	19	MSI-H	M	65	M	U	U	U	M	U	M	M	M	U	M	U	W	W
Lynch syndrome	1	HNPCC	M	40	U	U	U	M	U	M	U	M	U	U	M	U	W	W
	2	HNPCC	M	38	U	U	U	U	U	U	U	U	U	U	U	U	W	W
	5	HNPCC	F	65	U	U	U	U	M	U	U	U	U	U	M	M	M	W
	6	HNPCC	M	43	M	M	U	U	U	U	U	U	U	U	U	U	W	W
	7	HNPCC	M	50	M	U	U	U	M	U	M	M	U	U	M	M	W	W
	8	HNPCC	F	31	U	U	U	U	U	U	M	U	U	U	M	U	W	W
	9	HNPCC	F	34	U	U	U	U	U	U	M	U	U	U	M	U	W	W
	10	HNPCC	M	27	U	U	U	U	U	U	M	U	U	U	M	U	W	W
	13	HNPCC	F	25	U	U	U	U	U	U	U	U	U	U	M	U	M	W
	14	HNPCC	F	40	U	U	U	U	U	U	M	U	U	U	M	M	W	W
	15	HNPCC	M	53	U	U	U	U	U	U	U	M	U	U	M	U	W	W
	16	HNPCC	M	33	U	U	U	U	U	U	M	U	U	M	M	U	W	W
	17	HNPCC	M	54	U	U	U	U	U	U	U	U	U	U	M	M	M	W
	20	HNPCC	F	36	M	M	U	U	U	M	U	U	U	U	U	U	W	W
	22	HNPCC	M	56	U	U	U	U	U	U	U	U	U	U	U	M	M	W
	23	HNPCC	M	37	U	U	U	U	M	U	U	U	U	U	M	U	M	W

Figure 3. Methylation status of the genes in mismatch repair deficient colorectal cancers (sporadic and Lynch syndrome).

CIMP (+) results in Lynch syndrome CRCs. Among the non-CIMP markers, $p14^{ARF}$ showed a similar methylation pattern to the CIMP panel and showed methylation mainly in sporadic MSI-H CRCs. Almost no methylation was observed in either *DAPK* or *COX2* in Lynch syndrome, whereas infrequently methylation of both promoters was observed in sporadic MSI-H. In contrast, DCC was frequently methylated throughout the sporadic MSI-H and Lynch syndrome CRCs, whereas *MGMT* showed methylation less frequently overall but at similar levels in both types of CRCs.

Efficiency of molecular screening for Lynch syndrome from MSI-H colorectal cancer patients. As MSI-H tumors are characterized by MMR deficiency and include both Lynch syndrome and sporadic CRC, efficient elimination of sporadic CRC from total MSI-H CRC improves efficiency in diagnosing Lynch syndrome (Table I). Promoter methylation of the 3' region of *MLH1* (CCAAT methylation) was identified in 16 out of 19 sporadic MSI-H CRCs and 2 out of 16 Lynch syndrome cases. Thus if CCAAT non-methylation is considered a positive result for diagnosing Lynch syndrome, this corresponds to a sensitivity of 88% and specificity of 84%. Positive likelihood ratio (PLR) of non-CCAAT methylation among MSI-H CRC in predicting Lynch syndrome was 5.5 and negative likelihood ratio (NLR) of CCAAT methylation in predicting Lynch syndrome was 0.15. When the mutational status of *BRAF* instead of the CCAAT methylation is considered, sensitivity for the diagnosis of Lynch syndrome is 100% and the specificity is 84%. Thus, PLR of *BRAF* mutation among MSI-H CRCs to diagnose sporadic MSI-H CRCs was 6.3 and NLR of wild-type of *BRAF* to diagnose Lynch syndrome was zero. We also evaluate CIMP status using 5 markers as >3 markers being considered as positive for CIMP. Sensitivity for CIMP negative plus MSI-H to diagnosis Lynch syndrome was 88% and the specificity was 79%. PLR of CIMP negative for Lynch syndrome was 4.2 and NLR of CIMP positive for Lynch syndrome CRC was 0.16.

Discussion

Lynch syndrome is a dominantly inherited syndrome characterized by the development of CRC, endometrial cancer and other cancers and the presence of MSI in tumors. The Bethesda guidelines have been proposed to recruit families suspected of Lynch syndrome that require further molecular analysis (seeking germline mutation of MMR genes). In this study we evaluated how methylation status of *MLH1*, CIMP promoters and mutational status of *BRAF* V600E would help to improve selection of suspected Lynch syndrome patients before germline analysis.

The Bethesda guideline and revised Bethesda guideline are selection criteria designed to improve the probability of finding of Lynch syndrome cases by applying the MSI test. The recommended reference panel, referred to as the Bethesda

Table I. Sensitivity and specificity of Lynch syndrome screening with *MLH1* CCAAT methylation and *BRAF* V600E mutation among colorectal cancer with MSH-H.

	Lynch	Sporadic	Sensitivity (LCL-UCL)	Specificity (LCL-UCL)	PLR (LCL-UCL)	NLR (LCL-UCL)
CCAAT meth (-)	14	3	88 (63-98)	84 (61-96)	5.54 (2.1-14.7)	0.15 (0-0.5)
CCAAT meth (+)	2	16	88 (63-98)	84 (61-96)	5.54 (2.1-14.7)	0.15 (0-0.5)
BRAF wt	16	3	100 (80-100)	84 (61-96)	6.33 (2.4-16.9)	0.00 (0-0.3)
BRAF mt	0	16	100 (80-100)	84 (61-96)	6.33 (2.4-16.9)	0.00 (0-0.3)
CIMP (-)	14	4	88 (63-98)	79 (55-92)	4.16 (1.8-9.8)	0.16 (0-0.6)
CIMP (+)	2	15	88 (63-98)	79 (55-92)	4.16 (1.8-9.8)	0.16 (0-0.6)

PLR, positive likelihood ratio; NLR, negative likelihood ration; LCI, lower confidence intervals; UCI, upper confidence intervals; CCAAT meth, MLH1 promoter methylation at CCAAT box; BRAF wt, wild-type for BRAF; BRAF mt, mutation for BRAF and CIMP, CpG island methylator phenotype.

panel, was proposed and broadly used to elucidate the tumors caused by MMR deficiency (23). In other words, the Bethesda guideline aims to eliminate sporadic CRC with MMR deficiency by using pattern recognition of the phenotypic cancer features expressed in a family before applying the MSI test. Another option to eliminate sporadic CRC with MMR deficiency is to use molecular tests that define the alterations 'typically' (more preferably 'specifically') observed in either hereditary or sporadic CRC with MMR defects after selection by the MSI test.

Promoter hypermethylation of the *MLH1* gene is considered to be associated with absence of expression of the MLH1 protein and causes sporadic CRCs with MSI-H (8,24-26). However, difficulty in promoter analysis of *MLH1* has been demonstrated in many studies because the methylation pattern in the *MLH1* promoter is unique. *MLH1* has few CpG islands in its promoter, especially on the 3' side, and most of the methylation dense area, located upstream of the promoter, has been shown to have no relation to the silencing of *MLH1* (19). Conversely, methylation at a small 3' region closer to the transcription start site invariably correlates with the absence of *MLH1* expression in colorectal cell lines and surgically removed materials (27). Deng *et al* identified a CCAAT box in this region, which specifically bound transcription factor CBF (28). Methylation of a CpG island located two base pairs upstream of the CCAAT box inhibits the binding of CBF to the CCAAT box. We have shown that this unique methylation is well correlated with the silencing of *MLH1* using our unique methylation-specific PCR after confirming the methylation pattern by bisulfite sequencing throughout the promoter. In this study, we assessed the usefulness of this methylation (termed CCAAT methylation) to recruit Lynch syndrome cases in comparison with the mutation status of *BRAF* V600E or the CIMP status of the tumor.

Sensitivity and specificity to diagnose Lynch syndrome by a combination of MSI-H and either CCAAT methylation or *BRAF* mutation were similar, as the sensitivities were 88 and 100% and the specificity of both was 84%.

Probability P of having a disease is related to odds O of having the disease by the simple equation 0 = P/1-P. If the fraction of MSI-H cases that are Lynch syndrome is estimated to be ~X% then the probability that a case is Lynch syndrome based only on its being MSI-H is X/100 and the odds of being Lynch syndrome are: X/100-X. Thus, a person with a probability of having Lynch syndrome of X% before the CCAAT methylation test has odds of X/100-X of having Lynch syndrome. After a positive test result for CCAAT methylation, the odds change to 5.5X/100-X. Because, 0 = P/1-P $\Rightarrow$ P = 0/0+1, the probability after the test result is P = 5.5X/4.5X+100.

CCAAT methylation at *MLH1* was identified as being specific to the sporadic MSI-H CRCs with the exception of 3 cases. All three cases lacked the mutation of *BRAF* and did not belong to the CIMP phenotype. Two of them lacked the IHC staining of MLH1. Although they did not meet the Amsterdam Criteria, some of them may belong to Lynch syndrome cases whose family history was not sufficient to meet the clinical criteria, even though there was elderly onset of cancer. On the other hand CCAAT methylation was identified in 2 cases of Lynch syndrome and neither case showed a confirmed germline mutation of the *MLH1* gene. Whether silencing of *MLH1* is caused by methylation of both alleles or by hemi-methylation (second hit) plus an un-identified germline mutation (first hit) of *MLH1* is unknown. Both of the cases were wild-type in both *KRAS* and *BRAF* and did not show CIMP. Of course CIMP can be applied as an additional marker to select Lynch syndrome among the MSI-H CRCs, although the method to assess CIMP is more complicated than the single methylation assay of *MLH1* CCAAT. In addition, sensitivity and specificity of CIMP to diagnose Lynch syndrome were not superior to either *BRAF* mutation or *MLH1* methylation.

In this study, primary selection of MMR deficient CRCs was not done by IHC study of the MMR proteins. Thus, we cannot assess how IHC staining improves the diagnosis of Lynch syndrome. Also, the current MSI test has several problems, as false positive and false negative cases based on results of the NCI panel have been identified. For example, Lynch syndrome caused by the germline mutation of *MLH6* infrequently shows MSI-H by the Bethesda panel markers. By using multiple mononucleotide markers, *MLH6* mutants can be properly diagnosed as MSI-H. In this study series, MSI-H

status was assessed by the classical Bethesda panel, so that several tumors with MMR defects including those with *MSH6* defects could have been missed.

Accordingly, if a more precise MSI test to define CRC with MMR can be achieved, MSI plus a molecular test including *BRAF* mutation and CCAAT methylation of *MLH1* could be a good strategy to narrow down the possible Lynch syndrome candidates, or could even substitute for the final test, since the ultimate examination of germline mutation of MMR genes does not always show mutation in Lynch syndrome patients. *BRAF* V600E could be the most cost effective and efficient pre-selection molecular strategy targeting CRCs with MSI-H.

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