

# Metastatic Pattern of Stage IV Colorectal Cancer with High-Frequency Microsatellite Instability as a Prognostic Factor

KENJI FUJIYOSHI<sup>1,2</sup>, GOU YAMAMOTO<sup>1</sup>, TAKASHI TAKENOYA<sup>3</sup>, AKEMI TAKAHASHI<sup>1</sup>,  
YOSHIKO ARAI<sup>1</sup>, MINA YAMADA<sup>1</sup>, MIHO KAKUTA<sup>1</sup>, KENSEI YAMAGUCHI<sup>4</sup>,  
YOSHITO AKAGI<sup>2</sup>, YOJI NISHIMURA<sup>3</sup>, HIROHIKO SAKAMOTO<sup>3</sup> and KIWAMU AKAGI<sup>1</sup>

<sup>1</sup>Division of Molecular Diagnosis and Cancer Prevention,

<sup>3</sup>Division of Gastroenterological Surgery, Saitama Cancer Center, Saitama, Japan;

<sup>2</sup>Department of Surgery, Kurume University, Fukuoka, Japan;

<sup>4</sup>Department of Gastrointestinal Oncology,

Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan

**Abstract.** *Background:* A recent clinical trial on the immune check-point inhibitor pembrolizumab demonstrated that microsatellite instability (MSI) is a good biomarker for response to this inhibitor. However, clinicopathological features of advanced colorectal cancer (CRC) with high-frequency MSI (MSI-H) are unclear. *Patients and Methods:* A total of 2,439 surgically resected CRC tissues were analyzed for MSI status, and mutational status of V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog (KRAS), neuroblastoma RAS viral oncogene homolog (NRAS) and v-Raf murine sarcoma viral oncogene homolog B (BRAF). Stage IV cases were selected, and clinical and molecular features were evaluated. *Results:* There was no significant survival difference observed between MSI-H CRC and microsatellite-stable (MSS) CRC in patients with stage IV disease (3.92 vs. 2.50 years;  $p=0.766$ ). However, hematogenous and lymphogenous metastasis-dominant CRC with MSI-H demonstrated poor prognosis, whereas peritoneal metastasis-dominant CRC with MSI-H demonstrated good prognosis, (1.33 vs. 5.2 years;  $p=0.006$ ). *Conclusion:* Prognosis of stage IV CRC with MSI-H depended on the metastatic pattern. These findings provide useful information for the adaptation of CRC immunotherapy.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and frequent cause of cancer-related death worldwide (1). Specifically, the 5-year survival rate of patients with advanced CRC is less than 30%, and further advances in chemotherapy and surgical techniques are required to improve the survival rate of patients with CRC. Recently, novel molecular-targeting drugs, such as antibody to epidermal growth factor receptor (EGFR), are based on the molecular mechanism of carcinogenesis and achieve good efficacy. However, biomarkers are often required for appropriate therapy and are predicted based on the molecular mechanism of carcinogenesis.

During carcinogenesis, genetic changes, including deletion, insertion and substitution of nucleotides, rearrangement and epigenetic changes are accumulated. At least two types of genetic changes are known: chromosomal instability (CIN) and microsatellite instability (MSI). CIN comprises of structural and numerical changes of chromosomes, whereas MSI comprises frequent mutations in the microsatellite area without obvious structural and numerical changes of chromosomes. It has been reported that in Western countries approximately 80% of CRCs exhibit CIN and 15% have MSI (2). MSI is caused by dysfunction of the mismatch repair system, which detects and repairs mismatches that occur during DNA replication (3, 4). CRCs with CIN and MSI demonstrate different clinicopathological features, probably because of different mechanisms of genetic changes (5).

Sporadic CRC with high-frequency MSI (MSI-H) is prone to develop in the proximal colon and in older females. Pathological characteristics are associated with the presence of tumour-infiltrating lymphocytes, a Crohn's-like lymphocytic reaction and mucinous/signet ring cell differentiation (6-9). Although CRC with MSI-H is indicative of good prognosis in stage II and III disease (10-12), several reports suggest that the prognosis of CRC with MSI-H is poorer than that of

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*Correspondence to:* Kiwamu Akagi, MD, Ph.D., Division of Molecular Diagnosis and Cancer Prevention, Saitama Cancer Center, 780 Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806, Japan. Tel: +81 487221111, Fax: + 81 487221129, e-mail: akagi@cancer-c.pref.saitama.jp

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microsatellite-stable (MSS) CRC in stage IV (13, 14), and is still unclear whether this is correct or not.

In a recent study, the immune checkpoint inhibitor pembrolizumab significantly prolonged the disease-free and overall survival (OS) rates for CRC with MSI-H compared to those for CRC with MSS (15). Therefore, immune checkpoint inhibitors are highly expected to be an effective treatment for CRC with MSI-H. In this study, we evaluated the clinicopathological and molecular characteristics of stage IV CRC with MSI-H which may become potential targets of immune checkpoint inhibitor therapy.

## Patients and Methods

**Patients and tissue samples.** The present study was conducted using 2,439 surgically resected primary CRC cases at the Saitama Cancer Center from October 1999 to March 2014. Patients who had a history of preoperative radiotherapy or chemotherapy, inflammatory bowel disease, or of familial adenomatous polyposis were excluded. Pathological staging was performed according to the tumour, node and metastasis (TNM) classification system (seventh edition) (16). Patients were followed-up until death or survival until June 2015. All tumour samples were analysed after obtaining informed consent from each patient. Tumour tissues were paired with normal colorectal tissues and stored at  $-80^{\circ}\text{C}$ . The present study was approved by the Ethics Committee of the Saitama Cancer Center (no. 476). All procedures performed in this study were conducted in accordance with the ethical standards of the Institutional and National Research Committees and with the 1964 Helsinki Declaration and its later amendments.

**Mutation analysis.** Genomic DNA was extracted from the fresh frozen tissue samples using the standard phenol-chloroform extraction method and assayed for mutation in V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog (*KRAS*), neuroblastoma *RAS* viral oncogene homolog (*NRAS*) and v-Raf murine sarcoma viral oncogene homolog B (*BRAF*). *KRAS* mutations in exon 2, 3 and 4, and *NRAS* mutation in exon 2 and 3 were detected by denaturing gradient gel electrophoresis or high resolution melting (HRM) analysis using a Rotor-Gene Q (Qiagen, Hilden, Germany), as described previously (17,18).

*BRAF* mutations in exon 15 (codon 600) were detected by polymerase chain reaction (PCR)-restriction fragment length polymorphism or HRM, as previously described (19).

**Analysis of microsatellite status.** The MSI analysis was performed using fluorescence-based PCR, as described previously (8). The MSI status was determined using five Bethesda markers: BAT25, BAT26, D5S346, D2S123 and D17S250. The MSI status was graded as MSI-H with the presence of two or more unstable markers, as MSI-low (MSI-L) with only one unstable marker, and as MSS with no unstable marker. MSI-positive markers were re-examined at least twice to confirm the results. MSI-L was grouped with MSS in this study.

**Analysis of *mutL* homolog 1 (*MLH1*) promoter hypermethylation.** All CRCs with MSI-H were analyzed for *MLH1* promoter hypermethylation. Methylation status was investigated by methylation-specific PCR, combined with bisulfite restriction analysis, as previously reported (19).

**Statistical analysis.** Possible associations between each mutation and the clinicopathological parameters of CRC were assessed using the Fisher's exact test for categorical variables and an independent *t*-test for the continuous variables. For this analysis, the OS time was calculated from either the date of surgery until the date of death from CRC, or the date of the last visit at follow-up (30 June 2015). The Kaplan–Meier method was used to estimate the distribution of OS, and the log-rank test to compare the distribution of survival time. All *p*-values were calculated using a paired test and *p*-values of less than 0.05 were considered statistically significant. All statistical analyses were performed with SPSS Statistics v.20 (SPSS, Inc., IBM Corp., Armonk, NY, USA).

## Results

**Patient characteristics of stage IV CRC.** The frequency of MSI-H was 5.9% in all cases and 3.7% in stage IV, respectively (Table I). The frequency of MSI-H was 8.9% in stage II, the highest among all the stages (Table II). *KRAS* mutation was detected in 42.6%, *NRAS* mutation in 3.1% and *BRAF* mutation in 4.6% of all CRC specimens (Table I). Although frequency of *KRAS* and *NRAS* mutation remained unchanged in each stage, the frequency of *BRAF* mutation increased from 3.4% at stage 0-I to 6.3% at stage IV ( $p=0.037$ ) (Table II).

**Clinicopathological and molecular characteristics of stage IV CRC with MSI-H.** Stage IV CRCs were categorized into two groups by MSI status. Stage IV CRCs with MSI-H were more frequently associated with proximal location ( $p<0.001$ ), large tumour size ( $p<0.001$ ), undifferentiated histological features ( $p<0.001$ ) and mucinous components ( $p=0.038$ ).

Furthermore, stage IV CRC with MSI-H exhibited a different metastatic pattern compared to that with MSS. CRCs with MSI-H were more frequently associated with the presence of peritoneal dissemination ( $p=0.017$ ) and less frequently associated with the presence of liver metastases ( $p=0.001$ ). The frequency of curative resection was significantly higher in patients with MSI-H than MSS CRC ( $p=0.030$ ). In addition, *BRAF* mutation was observed significantly more frequently in MSI-H ( $p=0.011$ ), whereas the frequency of the *KRAS* and *NRAS* mutations were not significantly different between CRC with MSI-H and that with MSS in stage IV (Table III).

**Survival analysis classified by MSI status in stage IV CRC.** There was no significant survival difference observed between patients with CRC with MSI-H and those with MSS CRC in stage IV [3.92 vs. 2.50 years; hazard ratio (HR)=0.892, 95% confidence interval (CI)=0.42-1.89,  $p=0.766$ ]. However, the survival curve for patients with CRC with MSI-H dropped sharply, intersected with that of CRC with MSS, and was stable after 4 years (Figure 1). This

Table I. Characteristics of patients with colorectal cancer (CRC).

	All CRC		Stage IV	
	n=2439	(%)	n=401	(%)
Gender				
Female	1017	(41.7)	170	(42.4)
Male	1422	(58.3)	231	(57.6)
Mean age at diagnosis $\pm$ SD, years	65.0 $\pm$ 10.3		63.4 $\pm$ 10.6	
Location				
C	192	(7.9)	45	(11.2)
A	317	(13.0)	64	(16.0)
T	216	(8.9)	37	(9.2)
D	76	(3.1)	10	(2.5)
S	622	(25.5)	111	(27.7)
RS	313	(12.8)	54	(13.5)
Ra•Rb•P	703	(28.8)	80	(19.9)
Location				
Proximal	725	(29.7)	146	(36.4)
Distal	1714	(70.3)	255	(63.6)
Mean tumor size $\pm$ SD, mm	45.6 $\pm$ 23.8		60.4 $\pm$ 26.6	
TNM stage				
0-I	546	(22.4)	–	–
II	762	(31.2)	–	–
III	730	(29.9)	–	–
IV	401	(16.4)	401	(100)
Histological feature				
Well	218	(8.9)	10	(2.5)
Moderate	2064	(84.6)	345	(86.0)
Poor	74	(3.0)	21	(5.2)
Mucinous	82	(3.4)	25	(6.2)
Signet	1	(0.1)	0	(0.0)
Mucinous component				
No	2136	(87.6)	328	(81.8)
Yes	303	(12.4)	73	(18.2)
MSI status				
MSS	2295	(94.1)	386	(96.3)
MSI-H	144	(5.9)	15	(3.7)
KRAS				
Wild-type	1369	(57.4)	211	(53.3)
Mutant	1016	(42.6)	185	(46.7)
ND	54	–	5	–
NRAS				
Wild-type	2313	(96.9)	381	(96.7)
Mutant	73	(3.1)	13	(3.3)
ND	53	–	7	–
BRAF				
Wild-type	2280	(95.4)	371	(93.7)
Mutant	109	(4.6)	25	(6.3)
ND	50	–	5	–

SD: Standard deviation, C: cecum, A: ascending colon, T: transverse colon, D: descending colon, S: sigmoidal colon, RS: rectosigmoid, Ra: rectum above the peritoneal reflexion, Rb: rectum below the peritoneal reflexion, P: proctos, Proximal: cecum to transverse colon, Distal: splenic flexure to sigmoidal colon, TNM: tumor-node-metastasis, well: well-differentiated tubular adenocarcinoma, moderate: moderately differentiated tubular adenocarcinoma, poor: poorly differentiated tubular adenocarcinoma, mucinous: mucinous adenocarcinoma, signet: signet ring cell carcinoma, ND: not determined.

suggests that there are two groups in CRC with MSI-H: one with poor prognosis and another with good prognosis. Univariate and multivariate survival analyses for CRC with MSI-H demonstrated that there was no statistical

significance, due to a small number of MSI-H cases. However, it did suggest that the metastatic pattern (liver metastasis- or peritoneal metastasis-dominant) was associated with survival in CRC with MSI-H (Table V).

Table II. Rates for mutation (mt) of v-Raf murine sarcoma viral oncogene homolog B (BRAF), V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) and neuroblastoma RAS viral oncogene homolog (NRAS), and for high-frequency microsatellite instability (MSI-H) by colorectal cancer stage.

	BRAF mt (%)	KRAS mt (%)	NRAS mt (%)	MSI-H (%)
Stage				
0-I	3.4	39.1	2.4	5.9
II	4.6	42.8	3.6	8.9
III	4.5	42.7	2.8	4.0
IV	6.3	46.7	3.3	3.7
Total	4.6	42.6	3.3	5.9

Survival analysis of patients with stage IV CRC classified by metastatic pattern and MSI status. We divided patients with stage IV CRC with MSI-H and MSS into two groups: i) a CRC with peritoneal metastasis-dominant group (PM); and ii) a CRC with hematogenous and lymphogenous metastasis-dominant group (HL). CRC with PM was defined as cases with peritoneal metastasis with fewer than four lymph node metastases. CRC with HL which included CRC with liver metastasis, lung metastasis, or more than three lymph node metastases was defined. Finally, stage IV CRCs were classified into four groups: i) MSI-H CRC with HL (MSI-H-HL); ii) MSI-H CRC with PM (MSI-H-PM); iii) MSS CRC with HL (MSS-HL); and iv) MSS CRC with PM (MSS-PM).

Patients with MSI-H-HL had the poorest OS rate of the four groups (vs. MSI-H-PM,  $p=0.006$ ; vs. MSS-PM,  $p=0.004$ ; vs. MSS-HL,  $p=0.021$ ). In the multivariate analysis, there was a significant decrease in the median OS rate for those with MSI-H-HL compared to those with MSS-HL (1.33 vs. 2.42 years; HR = 2.34, 95% CI=1.01-4.99;  $p=0.027$ ) and MSS-PM (1.33 vs. 5 years; HR = 4.03, 95% CI=1.47-11.04;  $p=0.007$ ). In contrast, all patients survived in the MSI-H-PM group, which demonstrated the best OS rate of the four groups (vs. MSI-H-HL,  $p=0.006$ ; vs. MSS-PM,  $p=0.08$ ; vs. MSS-HL,  $p=0.022$ ) (Figure 2).

Pathological and molecular characteristics of MSI-H-PM and MSI-H-HL. The frequency of KRAS mutation between MSI-H-PM and MSI-H-HL groups was not different ( $p=1.000$ ). In contrast, the frequency of BRAF mutation in MSI-H-HL cases (40%) tended to be higher than that in MSI-H-PM cases (0%) ( $p=0.231$ ), no MSI-H-PM case had BRAF mutation.

BRAF-mutated CRC with MSI-H demonstrated a poorer prognosis than wild-type BRAF CRC with MSI-H in stage IV (HR=1.575, CI=0.286-8.673;  $p=0.601$  vs. BRAF wild-type) (Figure 3). MLH1 promoter hypermethylation was observed in eight cases among 15 stage IV CRCs with MSI-H, consisting of two MSI-H-PM and six MSI-H-HL cases

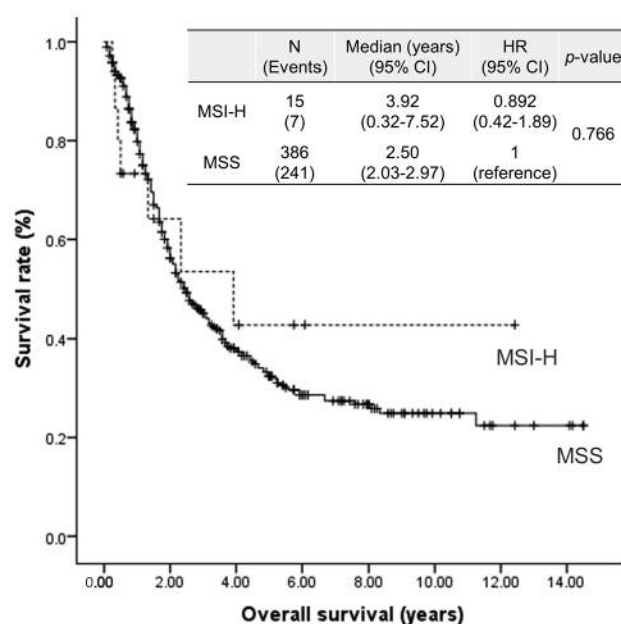


Figure 1. Survival analysis of patients with stage IV colorectal cancer classified by microsatellite instability (MSI) status. There was no significant survival difference for those with high-frequency MSI (MSI-H) CRC compared to those with microsatellite stable (MSS) CRC. HR: Hazard ratio, CI: confidence interval.

(Table IV). OS rates were not significantly different between hypermethylated MLH1 promoter CRC with MSI-H and unmethylated CRC with MSI-H in stage IV (Figure 4).

In comparison between MSI-H and MSS, the frequency of BRAF mutation in MSI-H-HL cases was significantly higher than that in MSS-HL cases ( $p=0.002$ ). Moreover, the curability rate of MSI-H-HL tended to be higher than MSS-HL ( $p=0.120$ ). The curability rate of MSS-PM was significantly higher than that of MSS-HL ( $p=0.003$ ) (Table IV).

## Discussion

In this study, we found that there were two main groups in stage IV CRC with MSI-H, one with extremely good prognosis (MSI-H-PM), and one with a poor prognosis (MSI-H-HL). In general, CRC with MSI-H is a better prognostic indicator than MSS in stage II and III CRC (10-12), whereas no difference was observed between corresponding groups in stage IV CRC in our study (Figure 1). However, the Kaplan-Meier curve in Figure 1 indicates that stage IV CRC with MSI-H may consist of two different groups: i) a group with poor prognosis, demonstrated by the curve dropping sharply during the early phase; and ii) a group with long-term survival. The univariate and multivariate analyses suggest that the metastatic patterns do indeed affect prognosis (Table V). Several reasons can be

Table III. Characteristics of patients with stage IV colorectal cancer.

	MSI-H		MSS		<i>p</i> -Value
	n=15	(%)	n=386	(%)	
Gender					
Female	9	(60.0)	161	(41.7)	0.188 <sup>†</sup>
Male	6	(40.0)	225	(58.3)	
Mean age at diagnosis $\pm$ SD, years	60.0 $\pm$ 17.5		63.5 $\pm$ 10.2		0.424 <sup>‡</sup>
Location					
C	2	(13.3)	43	(11.1)	0.017 <sup>†</sup>
A	7	(46.7)	57	(14.8)	
T	3	(20.0)	34	(8.8)	
D	1	(6.7)	9	(2.3)	
S	1	(6.7)	110	(28.5)	
RS	0	(0.0)	54	(14.0)	
Ra•Rb•P	1	(6.7)	79	(20.5)	
Location					
Proximal	12	(80.0)	134	(34.7)	<0.001 <sup>†</sup>
Distal	3	(20.0)	252	(65.3)	
Mean tumor size $\pm$ SD, mm	89.0 $\pm$ 36.5		59.3 $\pm$ 25.5		<0.001 <sup>‡</sup>
Histological feature					
Well	0	(0.0)	10	(2.6)	<0.001 <sup>†</sup>
Moderate	8	(53.3)	337	(87.3)	
Poor	5	(33.3)	16	(4.1)	
Mucinous	2	(13.3)	23	(6.0)	
Signet	0	(0.0)	0	(0.0)	
Mucinous component					
No	9	(60.0)	319	(82.6)	0.038 <sup>†</sup>
Yes	6	(40.0)	67	(17.4)	
Nodal metastases					
N $\leq$ 3	10	(66.7)	235	(60.9)	0.790 <sup>†</sup>
N $\geq$ 4	5	(33.3)	151	(39.1)	
Lung metastases					
No	15	(100.0)	320	(82.9)	0.146 <sup>†</sup>
Yes	0	(0.0)	66	(17.1)	
Liver metastases					
No	11	(73.3)	112	(29.0)	0.001 <sup>†</sup>
Yes	4	(26.7)	274	(71.0)	
Peritoneal metastases					
No	6	(40.0)	276	(71.5)	0.017 <sup>†</sup>
Yes	9	(60.0)	110	(28.5)	
P1	(4)	(44.4)	(46)	(41.8)	
P2	(5)	(55.6)	(35)	(31.8)	
P3	(0)	(0.0)	(29)	(26.4)	
Curability					
R0	13	(86.7)	216	(56.0)	0.030 <sup>†</sup>
R1	2	(13.3)	170	(44.0)	
KRAS					
Wild-type	10	(66.7)	201	(51.9)	0.307 <sup>†</sup>
Mutant	5	(33.3)	180	(48.1)	
ND	0	-	5	-	
NRAS					
Wild-type	15	(100.0)	366	(96.6)	1.00 <sup>†</sup>
Mutant	0	(0.0)	13	(3.4)	
ND	0	-	7	-	
BRAF					
Wild-type	11	(73.3)	360	(94.5)	0.011 <sup>†</sup>
Mutant	4	(26.7)	21	(5.5)	
ND	0	-	5	-	

MSI-H: High-frequency microsatellite instability; MSS: microsatellite stable; SD: standard deviation, C: cecum, A: ascending colon, T: transverse colon, D: descending colon, S: sigmoidal colon, RS: rectosigmoid, Ra: rectum above the peritoneal reflexion, Rb: rectum below the peritoneal reflexion, P: proctos, Proximal: cecum to transverse colon, Distal: splenic flexure to sigmoidal colon, well: well-differentiated tubular adenocarcinoma, moderate: moderately differentiated tubular adenocarcinoma, poor: poorly differentiated tubular adenocarcinoma, signet: signet ring cell carcinoma, mucinous: mucinous adenocarcinoma, P1: presence of nodules at proximal peritoneum, P2: few nodules at distal peritoneum, P3: many nodules at distal peritoneum, R0: no cancer remnant, R1: presence of cancer remnant, BRAF: v-Raf murine sarcoma viral oncogene homolog B, KRAS: V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog, NRAS: neuroblastoma RAS viral oncogene homolog, ND: not determined. <sup>†</sup>Fisher's exact test, <sup>‡</sup>independent *t*-test.

Table IV. Molecular and pathological features of each type of metastasis according to microsatellite instability (MSI) status in colorectal cancer (CRC).

	MSI-H				MSS			
	MSI-H-PM		MSI-H-HL		MSS-PM		MSS-HL	
	%	(mt/Total)	%	(mt/Total)	%	(mt/Total)	%	(mt/Total)
KRAS-mutant	40.0	(2/5)	30.0	(3/10)	48.0	(12/25)	47.2 <sup>a</sup>	(168/356)
NRAS-mutant	0.0	(0/5)	0.0	(0/10)	8.3	(2/24)	2.9	(11/355)
BRAF-mutant	0.0	(0/5)	40.0 <sup>b</sup>	(4/10)	12.0 <sup>c</sup>	(3/25)	5.1	(18/356)
MLH1 methylation	40.0	(2/5)	60.0	(6/10)	-	-	-	-
Curability (R0)	100.0	(5/5)	80.0 <sup>d</sup>	(8/10)	84.0	(21/25)	54.0 <sup>e</sup>	(195/361)

BRAF: v-Raf murine sarcoma viral oncogene homolog B, KRAS: V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog, NRAS: neuroblastoma RAS viral oncogene homolog, *MLH1*: mutL homolog 1, *MLH1* methylation: *MLH1* promoter hypermethylation, R0: no cancer remnant, MSS: microsatellite stable, MSI-H: high-frequency microsatellite instability, PM: peritoneal metastasis-dominant CRC, HL: hematogenous and lymphogenous metastasis-dominant CRC. <sup>a</sup>vs. MSS-PM:  $p=0.835$ , vs. MSI-H-HL:  $p=0.347$ ; <sup>b</sup>vs. MSS-HL:  $p=0.002$ , vs. MSS-PM:  $p=0.231$ ; <sup>c</sup>vs. MSS-HL:  $p=0.156$ ; <sup>d</sup>vs. MSI-H-PM:  $p=0.524$ , vs. MSS-HL:  $p=0.120$ , <sup>e</sup>vs. MSS-PM:  $p=0.003$ .

Table V. Univariate and multivariate overall-survival analysis in stage IV colorectal cancer with high-frequency microsatellite instability.

	Univariate			Multivariate		
	HR	95% CI	p-Value	HR	95% CI	p-Value
Location						
Proximal	36.173	0.033-39110	0.314	-	-	-
Distal	1	-	-			
Mucinous component						
Yes	0.871	0.296-1.55	0.358	-	-	-
No	1	-	-			
Liver metastases						
Yes	3.378	0.744-15.38	0.115	0.066	0.002-2.380	0.137
No	1	-	-			
PM						
Yes	1.011	0.191-5.347	0.99	-	-	-
No	1	-	-			
PM-dominant						
Yes	0.011	2.14E-5-5.681	0.157	193512	0.000-4.357E+269	0.969
No	1	-	-			
Curability						
R1	1.904	0.197-18.18	0.578	-	-	-
R0	1	-	-			
KRAS						
Mutant	0.847	0.163-4.385	0.843	-	-	-
Wild-type	1	-	-			
BRAF						
Mutant	1.574	0.286-8.695	0.601	-	-	-
Wild-type	1	0	-			

HR: Hazard ratio, CI: confidence interval, proximal: cecum to transverse colon, distal: splenic flexure to sigmoidal colon, PM: peritoneal metastases, R0: no cancer remnant, R1: presence of cancer remnant, KRAS: V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog, BRAF: v-Raf murine sarcoma viral oncogene homolog B.

considered for why MSI-H-HL in stage IV CRC was found to be associated with a poor prognosis. Firstly, *BRAF* mutation would adversely affect prognosis. It has been reported that *BRAF* mutation was a poor prognostic factor in

several retrospective studies (8, 20-22). Additionally, in CRC with MSI-H, *BRAF*-mutated CRC was also found to have poorer prognosis than CRC with wild-type *BRAF* (23). Similarly to these previous reports, our data show that

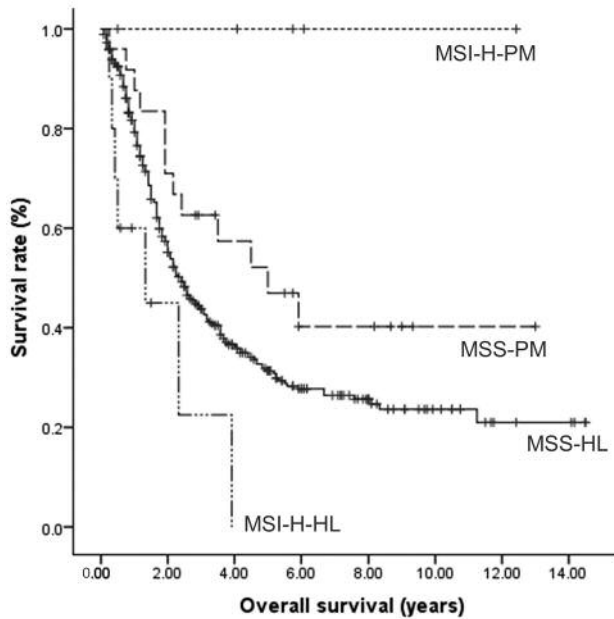


Figure 2. Survival analysis of patients with stage IV colorectal cancer CRC classified by metastatic pattern and microsatellite instability (MSI) status. Those with hematogenous/lymphogenous metastasis-dominant MSI-high (MSI-H-HL) CRC had the poorest overall survival out of the four groups [vs. peritoneal metastasis-dominant (MSI-H-PM):  $p=0.006$ ; vs. microsatellite stable (MSS):  $p=0.004$ ; vs. MSS-HL:  $p=0.021$ ]. All cases in the MSI-H-PM group survived and MSI-H-PM had the best overall survival rate out of the four groups (vs. MSI-H-HL:  $p=0.006$ ; vs. MSS-PM:  $p=0.08$ ; vs. MSS-HL:  $p=0.022$ ). HR: Hazard ratio, CI: confidence interval.

*BRAF*-mutated stage IV CRC with MSI-H demonstrated a poorer prognosis than that wild-type *BRAF*. Whereas, *BRAF* mutation with MSI-H was not associated with poorer OS than *BRAF* mutation with MSS in stage I-III CRC (24). Poor prognosis associated with *BRAF* mutation may be dependent on tumour stage. Furthermore, the frequency of *BRAF* mutation was 40% in MSI-H-HL compared to none in MSI-H-PM cases. Since not all cases of MSI-H-HL had the *BRAF* mutation, there may be factors of poor prognosis at play other than the *BRAF* mutation. In the future, we plan to investigate the mechanism of this association by

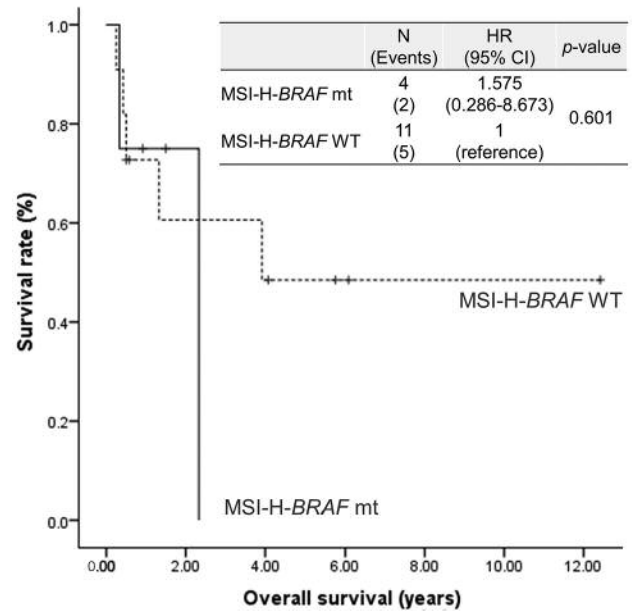


Figure 3. Survival analysis of patients with stage IV colorectal cancer classified by v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) mutation. Patients with *BRAF* mutation (mt) had a poorer prognosis compared to those with wild-type (WT) *BRAF*. HR: Hazard ratio, CI: confidence interval.

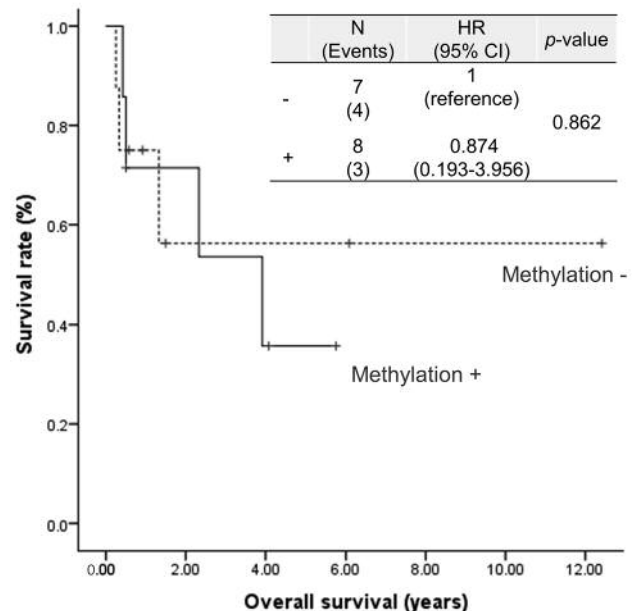


Figure 4. Survival analysis of patients with stage IV colorectal cancer classified by the methylation status of the mutL homolog 1 (*MLH1*) promoter. Survival of patients with hypermethylation of the *MLH1* promoter (+) was not significantly different from that of those without *MLH1* promoter hypermethylation (-). HR: Hazard ratio, CI: confidence interval.

accumulating more cases and examining multiple cancer-related genes.

In general, CRC with MSI-H exhibits a large tumour size and has high growth ability but less metastasis (18, 25, 26). Therefore, once these tumours acquire metastatic potential, the prognosis might worsen. From a recent large-scale genome analysis, Guinney *et al.* showed there to be four consensus molecular subtypes (CMS) of CRC (27). From this study, the CMS1 population, which tended to have hypermutated type (high number of somatic mutation events in the genome) and *BRAF* mutation, had a very poor survival rate after relapse, but good OS and relapse-free survival. A part of the CMS3 population tended to have hypermutated type and *KRAS* mutation. MSI-H-HL and MSI-H-PM in our study may correspond to the CMS1 and CMS3 populations, respectively.

In contrast with the poor prognosis of MSI-H-HL CRC, patients with MSI-H-PM CRC had a prolonged OS rate, and there were no deaths during the observation period. Vatandoust *et al.* reported that patients with PM CRC who underwent curative surgery could expect long-term survival (28). Similarly to Vatandoust *et al.*'s report, MSI-H-PM also conferred a better prognosis in patients with curatively resected stage IV CRC in our study.

The following are considered to be the reasons for why MSI-H-PM in stage IV CRC has a good prognosis. Firstly, MSI-H-PM cases did not have *BRAF* mutation, which is a poor prognostic factor. Even though *BRAF*-mutated CRC is likely to develop PM (29), no MSI-H-PM cases were *BRAF*-mutant. Moreover, the report that *BRAF*-mutated CRCs with MSI-H are less likely to exhibit peritoneal metastasis supports our results (30). Thus, our detailed analysis suggests that the frequency of *BRAF* mutations in PM is dependent on the MSI status. Additionally, curatively resected MSI-H-PM cases could be considered as having the same oncological status as during the early stage. Therefore, MSI-H-PM cases may maintain a better prognosis.

In this study, a multivariate analysis could not be performed sufficiently due to the small number of CRC cases with MSI-H. Frequency of CRC with MSI-H is approximately 10-15% in Western countries, whereas that in Asia was reported to be 5.5-7.3% (18, 31, 32), lower than in Western countries. However, despite the small number of cases, there was a significant prognostic difference between MSI-H-HL and MSI-H-PM cases. In a recent clinical trial, the immune checkpoint inhibitor, pembrolizumab prolonged disease-free and OS rates for metastatic CRC with MSI-H (15). Thus, MSI-H is an attractive biomarker for immunotherapy and these findings provide useful information for the adaptation of CRC immunotherapy. In conclusion, we found that the difference in metastatic pattern in stage IV CRC with MSI-H affects prognosis.

## Compliance with Ethical Standards

This study was approved by the Ethics Committee of the Saitama Cancer Center.

## Conflicts of Interest

The Authors declare that they have no competing of interest in regard to this study.

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