TABLE I. Correlation Between Clinicopathologic Variables and Combined MSI and *BRAF* Status

		MSI-H and *BRAF*-W		MSS and *BRAF*-W		MSS and *BRAF*-M		MSI-H and *BRAF*-M					
Variables	Total no.	No.	(%)	No.	(%)	No.	(%)	No.	(%)	*P* value[a]	*P* value[b]	*P* value[c]	*P* value[d]
Total No.	405	10		374		16		5					
Age (years)													
Mean ± SD	64.4 ± 9.9	56.8 ± 11.3		64.7 ± 9.5		58.8 ± 12.6		74.6 ± 6.2		0.010*	NS (0.675)*	0.018*	0.022*
<60	118	5	(50.0)	106	(28.0)	7	(43.7)	5	(100.0)	NS (0.160)	NS (>0.999)	NS (0.257)	0.002
>60	287	5	(50.0)	268	(72.0)	9	(56.3)	0	(0.0)				
Gender										NS (0.200)	NS (0.701)	NS (0.435)	NS (0.080)
Male	242	4	(40.0)	229	(60.7)	8	(50.0)	1	(25.0)				
Female	163	6	(60.0)	145	(39.3)	8	(50.0)	4	(75.0)				
Tumor location										NS (0.176)	NS (0.263)	NS (0.103)	NS (0.204)
Proximal	106	7	(70.0)	87	(24.3)	7	(43.7)	5	(100.0)	0.003[e]	NS (0.337)[e]	NS (0.053)[e]	0.004[e]
Distal	177	1	(10.0)	172	(45.4)	4	(25.0)	0	(0.0)				
Rectum	122	2	(20.0)	115	(30.3)	5	(31.3)	0	(0.0)				
Tumor histological grade										0.001	NS (0.692)	0.001	<0.001
Grade 1 or 2	372	6	(60.0)	354	(93.7)	11	(68.7)	1	(25.0)				
Grade 3 or 4	33	4	(40.0)	20	(6.3)	5	(31.3)	4	(75.0)				
pT stage										0.048**	0.005**	0.002**	NS (0.297)**
pT1 or pT2	73	4	(40.0)	64	(17.9)	1	(6.2)	0	(0.0)				
pT3	277	6	(60.0)	263	(69.4)	8	(50.0)	4	(75.0)				
pT4	55	0	(0.0)	47	(12.7)	7	(43.8)	1	(25.0)				
pN stage										NS (0.128)	NS (0.121)	NS (0.568)	NS (0.605)
pN1	303	10	(100.0)	279	(74.4)	11	(68.8)	3	(60.0)				
pN2	102	0	(0.0)	95	(25.6)	5	(31.2)	2	(40.0)				
Stage (7th AJCC)										0.034**	0.003**	0.031**	NS (0.118)**
IIIA	69	4	(40.0)	64	(16.9)	1	(6.3)	0	(0.0)				
IIIB	267	6	(60.0)	249	(66.5)	9	(56.2)	3	(60.0)				
IIIC	69	0	(0.0)	61	(16.6)	6	(37.5)	2	(40.0)				
Preoperative CEA (ng/ml)										NS (0.314)	NS (0.425)	NS (>0.999)	NS (>0.999)
Missing	1	0		1		0		0					
<5	269	5	(50.0)	249	(66.9)	11	(68.7)	4	(75.0)				
>5	135	5	(50.0)	124	(33.1)	5	(31.3)	1	(25.0)				
Preoperative CA19-9 (ng/ml)										NS (0.135)	NS (0.427)	<0.001	NS (0.502)
Missing	1	0		1		0		0					
<37	344	7	(70.0)	325	(87.0)	8	(50.0)	4	(75.0)				
>37	60	3	(30.0)	48	(13.0)	8	(50.0)	1	(25.0)				
LNR										NS (0.068)	NS (0.135)	NS (>0.999)	NS (>0.999)
<20	297	10	(100.0)	271	(72.6)	12	(75.0)	4	(75.0)				
>20	108	0	(0.0)	103	(27.4)	4	(25.0)	1	(25.0)				
Adjuvant chemotherapy										NS (0.101)	NS (0.664)	NS (0.513)	NS (0.236)
Absence	79	4	(40.0)	69	(18.7)	4	(25.0)	2	(40.0)				
Presence	326	6	(60.0)	305	(81.3)	12	(75.0)	3	(60.0)				
Adjuvant chemotherapy regimen										NS (0.514)	NS (0.333)	NS (0.625)	NS (>0.999)
5-FU monotherapy	291	5	(83.3)	271	(89.0)	12	(100.0)	3	(100.0)				
5-FU + L-OHP	35	1	(16.7)	34	(11.0)	0	(0.0)	0	(0.0)				

AJCC, 7th edition of the American Joint Committee on Cancer; Proximal, cecum to transverse colon; Distal, splenic flexure to sigmoid; LNR, lymph node ratio (ratio between metastatic and examined lymph nodes); L-OHP, oxaliplatin; MSS, microsatellite stable;MSI-H, microsatellite instability-high; *BRAF*-W, *BRAF*-wild type; *BRAF*-M, BRAF-mutation; Tumor histological grade 1 or 2, well or moderately differentiated; grade 3 or 4, poorly or undifferentiated;NS, not significant.

[a]*P* value between MSI-H and *BRAF*-W vs. MSS and *BRAF*-W.

[b]*P* value between MSI-H and *BRAF*-W vs. MSS and *BRAF*-M.

[c]*P* value between MSS and *BRAF*-W vs. MSS and *BRAF*-M.

[d]*P* value between MSS and *BRAF*-W vs. MSH and *BRAF*-M.

[e]*P* value between proximal vs. distal colon.

**unpaired Student *t*-test.

****Mann–Whitney *U*-test; the remainig variables, Fisher's exact test.

malignancy, preoperative chemotherapy or radiotherapy, and unavailability of MSI and *BRAF* status.

Adjuvant chemotherapy consisted of a 5-FU-based regimen (5-FU/leucovorin [LV] [22], Capecitabine [23], UFT/LV [24], or S-1 [25]) or a L-OHP and 5-FU (FOLFOX or XELOX) combination regimen [1,3]. Adjuvant chemotherapy was continued to completion at 6 months or until the patient exhibited recurrence, unacceptable toxicity or refusal, or was judged as inappropriate for adjuvant chemotherapy by the attending physicians. Patients who terminated adjuvant chemotherapy without known recurrence for less than 3 months were defined as receiving no adjuvant treatment.

All patients were followed up at least every 3 months for the first year and every 6 months thereafter for a total 5 years. Follow-up assessment involved medical history, physical examination, tumor markers evaluation (CEA and CA19-9 levels), and chest/abdominal computed tomography at least every 6 months. Recurrence was diagnosed on the basis of imaging and, if necessary, either cytologic analysis or biopsy performed. Clinicopathological data were obtained from the medical records of patients. Informed consent was obtained from all patients before sample collection. This study was approved by the Ethics Committee of the Saitama Cancer Center.

Analysis of MSI and BRAF Status

The five Bethesda markers (BAT25, BAT26, D5S346, D2S123, and D17S250) were used to analyze the MSI status of tumors in accordance with the National Cancer Institute guidelines [26]. Polymerase chain reaction (PCR) and subsequent analyses were performed as previously described [27]. Low-levels of MSI (MSI-L) was categorized as MSS, due to a lack of marked differences in patient outcome among previous studies [10,28,29].

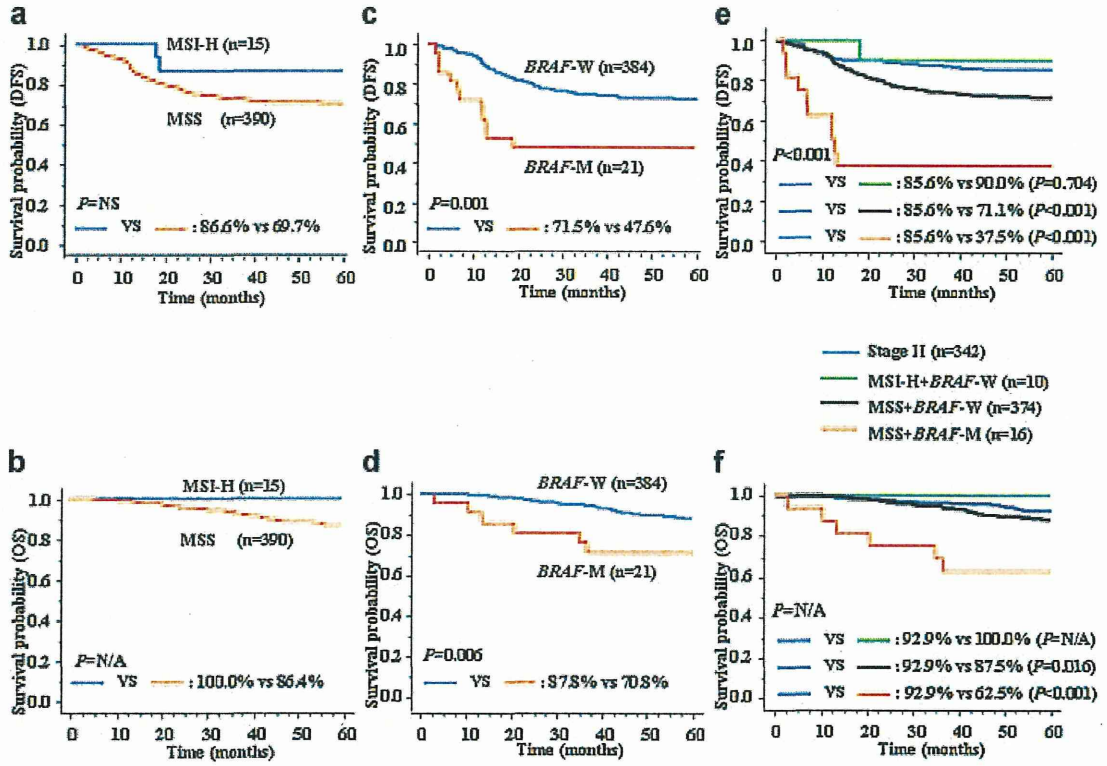

Fig. 1. Kaplan–Meier curves of 5-year DFS and OS in 405 stage III CRC patients. (a) DFS according to MSI status, (b) OS according to MSI status, (c) DFS according to *BRAF* mutational status, (d) OS according to *BRAF* mutational status, (e) DFS according to combined MSI and *BRAF* status, and (f) OS according to combined MSI and *BRAF* status. MSS, microsatellite stability; MSI-H, microsatellite instability-high; *BRAF*-W, *BRAF*-wild type; *BRAF*-M, *BRAF*-mutation; N/A, not applicable.

The *BRAF* V600E mutation, a hotspot in CRC, was examined using PCR combined with restriction enzyme digestion, as previously described [30]. All molecular marker data were analyzed by investigators completely blinded to patient identity and clinical and outcome data.

Statistical Analysis

Categorical variables were analyzed using Fisher's exact test or Mann–Whitney *U*-test, and continuous variables were analyzed using unpaired Student's *t*-test. For continuous variables, data are expressed as mean ± standard deviation (SD). Duration of follow-up was defined as time from tumor resection to death from any cause, last follow-up, or the cut-off date for this analysis (December 30, 2013). The Kaplan–Meier method was used to estimate the distributions of disease-free survival (DFS), recurrence-free survival (RFS), and overall survival (OS), and the log-rank test to compare distribution of survival time. Univariate and multivariate prognostic analyses were performed using the Cox proportional hazard model. $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were conducted using the Statistical Analysis System (SAS) software package (SAS Institute, Cary, NC, USA).

RESULTS

Patient Characteristics

This study consisted of 405 stage III CRC patients who underwent curative surgical resection with regional lymph nodes. Clinicopathological characteristics for the whole population are shown in Table I. The subgroups of stage III CRC consisted of 69 patients (17.0%) for stage IIIA, 267 (66.0%) for stage IIIB, and 69 (17.0%) for stage IIIC. A total of 326 patients (80.5%) received adjuvant chemotherapy, of which 291 (89.3%) were treated with a 5-FU-based regimen and 35 (10.7%) with a combination of L-OHP and 5-FU. The remaining 79 (19.5%) patients terminated adjuvant chemotherapy within 3 months due to unacceptable toxicity, patient refusal, or judgment by the attending physicians. With median follow-up of 57.3 months (range, 2.9–149.3 months), there were 115 events for DFS, 112 for RFS, and 44 for OS.

Association of MSI and BRAF Status With Clinicopathological Variables

Supplementary Table S1 summarizes clinicopathological characteristics of 405 patients based on MSI or *BRAF* status. MSS and MSI-H were detected in 390 (96.3%) and 15 (3.7%), respectively. *BRAF* mutations were observed in 21 (5.1%) of 405 patients. MSI-H was significantly associated with *BRAF* mutations ($P < 0.001$).

Clinical relevance of combined MSI and *BRAF* status was assessed in four groups, as follows: MSI-H and *BRAF*-wild type (n = 10), MSI-H and *BRAF*-mutation (n = 5), MSS and *BRAF*-wild type (n = 374), MSS and *BRAF*-mutation (n = 16). Compared with MSS and *BRAF*-wild type, the overall characteristics of the MSI-H and *BRAF*-wild type group were earlier subgroup of stage III, whereas those of the MSS and *BRAF*-mutant group were more advanced subgroup (Table I).

Association of MSI and BRAF Status With Prognosis

MSI and *BRAF* status were each examined for their prognostic value with DFS, RFS, and OS. In Kaplan–Meier analysis, the MSI-H phenotype showed non-significant trends toward better DFS and OS (Fig. 1a and b). Conversely, *BRAF*-mutations exhibited significantly worse DFS and OS than *BRAF*-wild type (Fig. 1c and d). *KRAS*-mutation had no influence on patient outcome for DFS, RFS, or OS (Supplementary Table S2).

Combined MSI and BRAF Status as Prognostic Marker

To determine whether the concomitant evaluation of both MSI and *BRAF* status provides an additive or subtractive effect on patient outcome (due to opposing effects), the association of MSI and *BRAF* status combination with prognosis was assessed. The combination of MSI and *BRAF* status provided significant prognostic stratification of DFS ($P < 0.001$, Fig. 1e). Further, MSI-H and *BRAF*-wild type was characteristic of stage II CRC from a prognostic point of view for DFS. Although prognostic analysis for OS could not be conducted, as no events were observed in the MSI-H and *BRAF*-wild type group, the combination of MSI and *BRAF* status showed potential as a prognostic marker (Fig. 1f). Although prognostic analysis of the MSI-H and *BRAF*-mutant group was deemed unreliable due to the small number of patients, outcomes of this group were similar to the MSS and *BRAF*-wild type group for DFS, RFS, and OS (Supplementary Fig. S1).

In multivariate prognostic analysis, the combination of MSI and *BRAF* status was independently associated with DFS ($P = 0.028$) and RFS ($P = 0.022$) in stage III CRC (Table II). Compared with the MSS and *BRAF*-wild type group, the MSS and *BRAF*-mutant group exhibited significantly worse DFS (HR, 2.35; 95% CI, 1.16 to 4.76; $P = 0.017$) and RFS (HR, 2.42; 95% CI, 1.19 to 4.91; $P = 0.014$). The MSI-H and *BRAF*-wild type group showed consistent trends toward better DFS (HR, 0.33; 95% CI, 0.04 to 2.51) and RFS (HR, 0.32; 95% CI, 0.04 to 2.45).

It remains to be determined whether or not the combination of MSI and *BRAF* status can confer additional prognostic information within each subgroup of stage III CRC (stage IIIA, stage IIIB, and stage IIIC). Multivariate prognostic analysis adjusting for subgroups of stage III CRC revealed that combined MSI and *BRAF* status remained an independent risk factor for prognosis in DFS and RFS (Supplementary Table S3). Next, the prognostic value of the combined status according to the subgroups of stage III CRC was assessed. Interestingly, patients in the *BRAF*-wild type group with stage IIIA CRC, irrespective of MSI status, shared characteristics with stage II CRC from a prognostic point of view in DFS, whereas the MSS and *BRAF*-mutant group exhibited a worse outcome (Fig. 2). In stage IIIB CRC, the MSI-H and *BRAF*-wild type group retained the characteristics of stage II CRC, whereas the MSS and *BRAF*-wild type group exhibited significantly worse outcomes than stage II CRC ($P < 0.001$), as did the MSS and *BRAF*-mutant group ($P < 0.001$). In stage IIIC CRC, MSS, irrespective of *BRAF* status, was no longer characteristic of stage II CRC ($P < 0.001$). These findings were also observed for RFS (Supplementary Fig. S2). The prognostic significance of MSI-H and *BRAF*-wild type in stage IIIC CRC could not be assessed due to case deletion. In Kaplan–Meier analysis for OS, MSS, irrespective of *BRAF* status, in stage IIIC CRC exhibited significantly worse outcomes than stage II CRC patients ($P < 0.001$; Supplementary Fig. S2). Further, similar trends were observed for DFS and OS even on separate analysis of 323 patients who treated with adjuvant chemotherapy (Supplementary Fig. S3).

TABLE II. Multivariate Prognostic Analysis in 405 Colorectal Cancer

		RFS			DFS		
Variables	Total no.	HR	(95% CI)	*P* value[a]	HR	(95% CI)	*P* value[a]
Tumor location				NS (0.080)			NS (0.069)
Proximal	106	1	(referent)		1	(referent)	
Distal	177	0.738	(0.439 to 1.241)	NS (0.251)	0.714	(0.425 to 1.198)	NS (0.202)
Rectum	122	1.266	(0.784 to 2.044)	NS (0.335)	1.313	(0.819 to 2.106)	NS (0.258)
Tumor histological grade				NS (0.178)			NS (0.193)
Grade 1 or 2	372	1	(referent)		1	(referent)	
Grade 3 or 4	33	1.532	(0.823 to 2.853)		1.509	(0.812 to 2.802)	
pT stage				0.046			0.011
pT1 or pT2	73	1	(referent)		1	(referent)	
pT3	277	1.668	(0.866 to 3.213)	NS (0.126)	1.855	(0.939 to 3.668)	NS (0.075)
pT4	55	2.542	(1.194 to 5.414)	0.015	3.100	(1.436 to 6.691)	0.003
pN stage				NS (0.281)			NS (0.167)
pN1	303	1	(referent)		1	(referent)	
pN2	102	1.291	(0.811 to 2.058)		1.379	(0.873 to 2.178)	
MSI and *BRAF* status				0.022			0.028
MSS and BRAF-W	374	1	(referent)		1	(referent)	
MSI-H and *BRAF*-W	10	0.324	(0.043 to 2.451)	NS (0.275)	0.333	(0.044 to 2.516)	NS (0.286)
MSS and *BRAF*-M	16	2.425	(1.195 to 4.919)	0.014	2.351	(1.161 to 4.762)	0.017
Preoperative CA19-9 (ng/ml)				0.035			0.049
<37	344	1	(referent)		1	(referent)	
>37	60	1.670	(1.036 to 2.691)		1.613	(1.002 to 2.596)	
LNR				0.027			0.049
<20	297	1	(referent)		1	(referent)	
>20	108	1.711	(1.060 to 2.763)		1.609	(1.001 to 2.586)	
Adjuvant chemotherapy				NS (0.057)			0.030
Absence	79	1	(referent)		1	(referent)	
Presence	326	0.643	(0.407 to 1.015)		0.610	(0.390 to 0.955)	

Proximal, cecum to transverse colon; Distal, splenic flexure to sigmoid; LNR, lymph node ratio (ratio between metastatic and examined lymph nodes); MSS, microsatellite stable; MSI-H, microsatellite instability-high; *BRAF*-W, *BRAF*-wild type; *BRAF*-M, *BRAF*-mutation; Tumor histological grade 1 or 2, well or moderately differentiated; grade 3 or 4, poorly or undifferentiated; NS, not significant.
[a]Cox proprtional harzard model.

Combined MSI and BRAF Status as a Predictive Marker for 5-FU-Based Chemotherapy

The effect of 5-FU-based adjuvant chemotherapy according to the combination of MSI and *BRAF* status was assessed (Supplementary Fig. S4). MSS, irrespective of *BRAF* status, exhibited favorable DFS in patients treated with 5-FU-based chemotherapy than in those who terminated adjuvant chemotherapy within 3 months. On the other hand, 5-FU-based chemotherapy was not associated with any improvement in either DFS or OS in the MSI-H and *BRAF*-wild type group.

The majority of patients treated with L-OHP and 5-FU-based chemotherapy were MSS and *BRAF*-wild type (except one patient with MSI-H and *BRAF*- wild type). The addition of L-OHP was therefore assessed in the MSS and *BRAF*-wild type group. As expected, L-OHP additively showed superior rates of DFS for both stage IIIB and IIIC CRC patients compared with 5-FU monotherapy (data not shown).

DISCUSSION

The identification of markers that are both prognostic and predictive of a response to therapy is indispensable for the establishment of a robust therapeutic strategy that maintains efficacy in line with currently available treatment regimens while reducing toxicity.

Subjects with the MSI-H phenotype generally had more favorable outcomes than those with the MSS phenotype, whereas *BRAF* mutations were significantly associated with poor outcomes, supporting the opposing prognostic effects of MSI-H and *BRAF* mutations (Fig. 1) [6–10,14–17,31,32]. When these molecular markers for prognostic risk were concomitantly assessed, the combination of MSI and *BRAF* status significantly exhibited prognostic stratification for DFS (Fig. 1E) and was independently associated with DFS and RFS in multivariate prognostic analysis (Table II), even when adjusted by subgroup of stage III CRC (Supplementary Table S3). This finding suggests that combination of MSI and *BRAF* status might be one of the most critical alterations to regulate an aggressive tumor phenotype and has potential as a prognostic marker to provide more accurate stratification of outcomes within the three subgroups of stage III CRC.

Although our findings must be interpreted with caution because of possible selection bias, MSS—irrespective of *BRAF* status—is likely to be beneficial from 5-FU-based chemotherapy (Supplementary Fig. S4). DFS in the MSS and *BRAF*-wild type group for stage IIIA CRC (but not stage IIIB or IIIC) were favorable, similar to those of stage II CRC patients (Fig. 2). Thus, in the MSS and *BRAF*-wild type group, 5-FU-based chemotherapy could reduce CRC from stage IIIA to stage II from a prognostic point of view, but its efficacy appears to be sufficient in stage IIIB and IIIC CRC. Addition of L-OHP showed improved outcomes for both stage IIIB and IIIC CRC. On the other hand, the MSS and *BRAF*-mutant group from stage IIIA CRC onwards did not exhibit characteristics similar to stage II CRC patients via the aggressive tumor phenotype. Of note, the MSI-H and *BRAF*-wild type group in stage III CRC patients had an excellent outcome despite receiving no benefit from 5-FU-based chemotherapy, an outcome similar to that of stage II CRC patients (Fig. 2 and Supplementary Fig. S4), for whom routine adjuvant therapy is not recommended [19]. This detrimental effect might allow subjects with this phenotype to avoid adjuvant chemotherapy. On considering both the estimated risk of recurrence and predictive efficacy from adjuvant chemotherapy (Fig. 2 and Supplementary Fig. S4), the combined MSI and *BRAF* status could categorize stage III CRC into the three subtypes to select the most effective treatments as follows (Table III): aggressive subtype, high risk of recurrence that would benefit most from addition of L-OHP (recommend therapy, combination of L-OHP, and 5-FU chemotherapy); moderate type, intermediate risk that would sufficiently benefit even without addition of L-OHP, similar outcome to stage II CRC patients (recommend therapy, 5-FU monotherapy); and defensive type, low risk that would potentially benefit from avoiding the cost, toxicity, and inconvenience of adjuvant chemotherapy in light of a lower likelihood of treatment benefit (recommend therapy, observation). Thus, the combined MSI and *BRAF* status might facilitate the establishment of personalized therapeutic strategies in adjuvant chemotherapy, such as determining the selection of the most suitable patients and adjuvant therapy regimen.

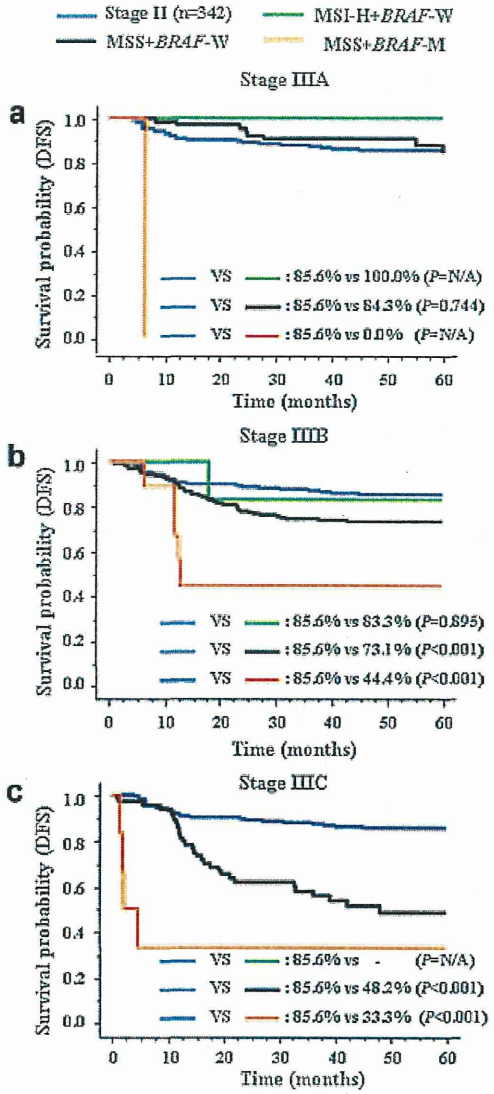

Figure 2. Kaplan–Meier curves of 5-year DFS in combined MSI and *BRAF* status according to subgroups of stage III CRC patients, compared with 342 stage II CRC patients. (a) Stage IIIA (n = 69), (b) Stage IIIB (n = 264) and (c) Stage IIIC (n = 67). *BRAF*-W, *BRAF*-wild type; *BRAF*-M, *BRAF*-mutation; N/A, not applicable.

Given that prognostic analysis of the MSI-H and *BRAF*-mutant group was deemed unreliable due to the small number of patients, the subtypes of the MSI-H and *BRAF*-mutant group remain elusive. In exploratory Kaplan–Meier analysis, DFS, RFS, and OS in the MSI-H and *BRAF*-mutant group were similar to those in the MSS and *BRAF*-wild type group (Supplementary Fig. S1), a finding consistent with those of recent studies suggesting that *BRAF*-mutant may somewhat influence favorable outcomes among patients with MSI-H [14,16,18].

TABLE III. Proposal Treapeutic Strategy for Ajuvant Chemotherapy for Stage III ater Curative Surgical Resection

Combined MSI and *BRAF* status	Stage IIIA	Stage IIIB	Stage IIIC
MSI-H and *BRAF*-W	observation (Defensive type)	observation (Defensive type)	?
MSS and *BRAF*-W	5-FU monotherapy (Moderate type)	5-FU + L-OHP (Aggressive type)	5-FU + L-OHP (Aggressive type)
MSS and *BRAF*-M	5-FU + L-OHP (Aggressive type)	5-FU + L-OHP (Aggressive type)	5-FU + L-OHP (Aggressive type)

MSS, microsatellite stable; MSI-H, microsatellite instability-high; *BRAF*-W, *BRAF*-wild type; *BRAF*-M, *BRAF*-mutation; L-OHP, oxaliplatin.
Aggressive subtype: high risk of recurrence that would benefit most from addition of L-OHP.
Moderate type: intermediate risk of recurrence that would sufficiently benefit even without addition of L-OHP, similar outcome to stage II CRC patients.
Defensive type: low risk that would potentially benefit from avoiding the cost, toxicity, and inconvenience of adjuvant chemotherapy in light of a lower likelihood of treatment benefit.

The MSI-H phenotype is more common in patients with stage II CRC (approximately 20%) than stage III (12%) or IV CRC (4%) [33]. Unexpectedly, MSI-H phenotype was observed in only 15 (3.7%) of the 405 patients with stage III CRC in the present study. Ethnic differences were reported in MSI status, which were more frequent in subjects of African American and Egyptian, whereas less in those of Korean [34–37]. The prevalence of the MSI-H phenotype might therefore be lower in Asia, including Japan or Korea.

The primary limitation to the present study is the small number of patients with MSI-H or *BRAF* mutation, which attenuated statistical powers on the analysis of combined MSI and *BRAF* status. In addition, both retrospective analyses and small number of patients who treated with a combination of L-OHP and 5-FU chemotherapy also have the potential weakness in the present study. In contrast, the strength of the present study is the homogeneous population with consecutive stage III CRCs that were diagnosed and treated at a single institution, reducing the impact of heterogeneity by various disease stages or institutions. In addition, in line with previous studies, we confirmed that the MSI-H phenotype exhibited unique characteristics, such as proximal colon predominant, high grade histology, frequent *BRAF* mutation, favorable outcome, and a lack of benefit from 5-FU adjuvant chemotherapy [6–10,38,39] and that *BRAF* mutant phenotype exhibited the unique characteristics as well, such as proximal colon predominant, high grade histology, and unfavorable outcome [14–17,40]. Thus, these consistent clinicopathological findings also support great clinical value of combined MSI and *BRAF* status in the present study. As the statistical power was quite limited and caution must be taken to interpret our findings, additional large studies are clearly needed to validate the clinical potential of combined MSI and *BRAF* status.

In conclusion, the clinical assessment of combined MSI and *BRAF* status serves as both a prognostic and predictive marker for stage III CRC, and this information might provide much-needed guidance during the planning of therapeutic strategies, such as determining the selection of the most suitable patients and adjuvant therapy regimen. Further research is required to validate the clinical potential of the MSI and *BRAF* status combination for stage III CRC.

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SUPPORTING INFORMATION

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Inverse Effect of Mucinous Component on Survival in Stage III Colorectal Cancer

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Background: Although mucinous adenocarcinoma (MAC) is has been recognized as a separate entity in colorectal cancer (CRC), adenocarcinoma with a mucinous component (ACM) remains poorly understood.
Methods: The association of MAC and ACM with disease-free survival (DFS) and overall survival (OS) was examined using the Cox proportional hazard model in 425 consecutive stage III CRCs.
Results: Compared with conventional adenocarcinoma (CAC), patients with MAC exhibited independently worse DFS (hazard ratio [HR], 2.64; 95% CI, 1.21–5.80; $P = 0.014$) and OS (HR, 3.56; 95% CI, 1.53–8.30; $P = 0.003$). Unexpectedly, ACM was significantly associated with worse OS than CAC ($P = 0.002$), despite having a similar DFS to CAC. Further, ACM patients after recurrence exhibited significantly worse OS than CAC patients ($P < 0.001$), similar to MAC.
Conclusions: Although ACM is similar to CAC with regard to estimated risk of recurrence, the outcome is extremely poor once recurrence occurs and is identical to MAC; one of the most aggressive phenotypes of stage III CRC. Thus, both MAC and ACM are adverse prognostic factors for OS. *J. Surg. Oncol.* © 2014 Wiley Periodicals, Inc.

KEY WORDS: **mucinous adenocarcinoma; mucinous component; colorectal cancer; stage III**

INTRODUCTION

Colorectal cancer (CRC) is the second-most common cancer and fourth leading cause of cancer-related death worldwide [1]. Although CRC screening programs such as faecal occult blood test and colonoscopy have reduced mortality, many patients have advanced disease at the time of diagnosis [2]. As many as 40%–50% of patients who undergo potentially curative surgery eventually relapse and die of metastatic disease [3]. Adjuvant chemotherapy has significantly decreased recurrence and mortality in patients with stage III CRC [4,5] and is recommended in the National Comprehensive Cancer Network (NCCN) guidelines [6].

Mucinous adenocarcinoma (MAC) is an uncommon histological subtype of adenocarcinoma (approximately 5%–15%) in CRC [7] and is defined by the World Health Organization (WHO) as adenocarcinoma with more than 50% extracellular mucin within the tumor [8]. Compared with conventional adenocarcinoma (CAC), MAC has unique biological entity, such as proximal colon predominance, advanced disease progression, frequent *BRAF* mutation, and unfavorable outcomes [9–11].

In contrast, adenocarcinoma with less than 50% extracellular mucin is categorized as adenocarcinoma with a mucinous component (AMC), but the clinicopathological features are poorly understood [12–15]. A recent study showed that ACM patients have significantly better outcomes than MAC ones, with similar disease-free survival (DFS) to CAC patients [14]. However, differences in the overall survival (OS) among MAC, AMC, and CAC remain elusive.

In the present study, we examined the outcomes of mucinous histology in stage III CRC patients following curative surgical resection.

MATERIALS AND METHODS

Patient Population

We evaluated a series of 425 consecutive patients with pathologically confirmed stage III CRC who underwent curative surgical resection with regional lymph node dissection at the Saitama Cancer Center from May 2001 to December 2011. Pathological TNM classification was assessed in accordance with the 7th edition of the AJCC staging system [16]. Exclusion criteria were patients with active concomitant malignancy and preoperative chemotherapy or radiotherapy.

Adjuvant chemotherapy consisted of a 5-FU-based regimen (5-FU/leucovorin [LV] [17], Capecitabine [18], UFT/LV [19], or S-1 [20]) or a combination regimen of oxaliplatin (L-OHP) and 5-FU (FOLFOX or XELOX) [4,5]. Adjuvant chemotherapy was continued to completion at 6 months or until the patient exhibited recurrence, unacceptable toxicity, or refusal, or was judged as inappropriate for adjuvant chemotherapy by attending physicians. Patients who terminated adjuvant chemotherapy without known recurrence for less than 3 months were defined as having received no adjuvant treatment.

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All patients were followed up at least every 3 months for the first year and every 6 months thereafter for a total of 5 years. Follow-up assessment involved medical history, physical examination, tumor marker evaluation (CEA and CA19-9 levels), and chest and abdominal computed tomography at least every 6 months and colonoscopy within 12 months after surgery. Recurrence was diagnosed on the basis of imaging and, if necessary, either cytology or biopsy was performed. Clinicopathological data were obtained from the medical records of patients. Informed consent was obtained from all patients before sample collection. This study was approved by the Ethics Committee of the Saitama Cancer Center.

Analysis of BRAF and KRAS Status

Of 425 tumor specimens, 405 were available for genetic analyses. The *BRAF* V600E mutation, a hotspot in CRC, was examined using polymerase chain reaction combined with restriction enzyme digestion, as previously described [21]. Mutations in exons 2 and 3 of the *KRAS* gene were analyzed by denaturing gradient gel electrophoresis, as previously described [22]. All molecular marker data were analyzed by investigators completely blinded to patient identity and clinical and outcome data.

Statistical Analysis

Categorical variables were analyzed using Fisher's exact test or Mann-Whitney *U*-test, and continuous variables were analyzed using unpaired Student's *t*-test. For continuous variables, data are expressed as mean ± standard deviation (SD). Duration of follow-up was defined as time from tumor resection to death from any cause, last follow-up, or the cut-off date for analysis (December 30, 2013). DFS was defined as time from tumor resection to recurrence or death from any cause, and OS was the time from tumor resection to death from any cause. Patients with no events at the cut-off date or alive for more than 5 years from tumor resection were censored on the closing date of the study or after 5 years, respectively. The Kaplan-Meier method was used to estimate the distribution of DFS and OS, and the log-rank test to compare distribution of survival time. Univariate and multivariate prognostic analyses were performed using the Cox proportional hazard model. $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were conducted using the Statistical Analysis System (SAS) software package (SAS Institute, Cary, NC, USA).

RESULTS

Patient and Clinicopathological Characteristics of Mucinous Histology

This study consisted of 425 stage III CRC patients who underwent curative surgical resection with regional lymph nodes. Median age was 64 years (mean ± SD, 64.1 ± 10.1 years). Adjuvant chemotherapy was administered to 345 patients (81.2%), of which 308 (89.3%) were treated with a 5-FU-based regimen and 37 (10.7%) with a combination of L-OHP and 5-FU. The remaining 80 (18.8%) patients terminated adjuvant chemotherapy within 3 months due to unacceptable toxicity, patient refusal, or judgment of the attending physicians. With median follow-up of 57.3 months (range, 2.9–155.2 months), there were 120 events for DFS and 46 for OS.

Table I summarizes the clinicopathological characteristics of the 425 patients based on mucinous histology. The following rates of adenocarcinoma were observed in the 425 patients: CAC in 371 (87.3%), ACM in 38 (8.9%), and MAC in 16 (3.8%) patients (Fig. 1). Compared with CAC, MAC was significantly associated with an advanced T-stage ($P = 0.023$) and had a higher incidence in men ($P = 0.018$). *BRAF* mutations tended to occur at a higher frequency in MAC and ACM patients than in CAC patients (*BRAF* mutation: 20.0% vs. 11.4% vs. 3.9%, respectively). No significant clinicopathological differences were observed between MAC and ACM patients.

Association of Mutinous Histology with Prognosis for DFS

In Kaplan-Meier analysis, 5-year DFS was 37.5% for MAC, 65.8% for ACM, and 72.5% for CAC (Fig. 2). The results of univariate analysis are shown in Table II. Patients with MAC exhibited significantly worse DFS than those with CAC ($P < 0.001$) but not those with ACM ($P = 0.72$).

Multivariate analysis was conducted to estimate survival hazard ratio (HR) based on the mucinous histology (Table II). Mucinous histology was independently associated with DFS ($P = 0.041$). Compared with CAC, MAC retained its prognostic impact for DFS (HR, 2.64; 95% CI, 1.21–5.80; $P = 0.014$). In addition, multivariate prognostic analysis adjusting for three subgroups of stage III (stage IIIA, stage IIIB, stage IIIC) revealed that the MAC remained an independent risk factor for prognosis (Supplementary Table I).

We then analyzed sites of first recurrence in patients with stage III CRC. Compared with the CAC, neither MAC nor ACM correlated with hematogenous recurrence, lymph nodes recurrence, or peritoneal recurrence (Supplementary Table II).

Seventy-four of 118 patients with recurrence underwent surgery with curative intent. On examining pathological concordance of mucinous component between primary and corresponding recurrent tumor in the 74 patients, six (85.7%) of seven patients with MAC and four (57.1%) of seven with ACM in the primary tumor exhibited identical mucinous histology in the recurrent tumor. In addition, only one of one patients with ACM in primary tumor exhibited MAC in recurrent tumor (Supplementary Table III).

Association of Mucinous Histology with Prognosis for OS

In Kaplan-Meier analysis, 5-year OS was 67.7% for MAC and 70.2% for ACM vs. 89.0% for CAC (Fig. 3A). In univariate analysis, MAC exhibited significantly worse OS than CAC ($P = 0.004$). Unexpectedly, ACM also had a significantly worse impact on OS than CAC ($P = 0.004$), despite similar DFS between the two (Table II).

To determine whether or not outcomes after recurrence reflect the unfavorable OS for ACM vs. CAC, prognostic analysis for OS—defined as time from tumor recurrence to death—was conducted for 118 patients with recurrence. ACM after recurrence exhibited significantly poorer OS than CAC ($P < 0.001$), similar to the outcome for MAC (Fig. 3B).

In multivariate analysis, MAC was significantly associated with worse OS compared with the CAC (HR, 3.56; 95% CI, 1.53–8.30; $P = 0.003$; Table II) and remained an independent risk factor for prognosis even after adjusting for the three subgroups of stage III (Supplementary Table I). Although there was no statistically significant difference, ACM patients tended to have unfavorable OS (HR, 2.31; 95% CI, 0.77–6.93; $P = 0.13$).

The relative effect of adjuvant chemotherapy for mucinous histology was assessed. The MAC and ACM exhibited a trend toward a beneficial effect in patients treated with adjuvant chemotherapy compared to those without adjuvant chemotherapy (Supplementary Fig. 1). However, this finding must be interpreted with caution due to small number of patients with mucinous histology and retrospective study.

DISCUSSION

While a number of studies have highlighted the clinicopathological features of MAC in CRCs of various disease stages, little is known of stage III CRC, with outcomes of ACM patients particularly poorly understood [12,14,15]. We therefore examined the estimated risk of OS according to the subtypes of mucinous histology of stage III CRC.

TABLE I. Clinicopathologic Correlation with Mucinous Histology in 425 Colorectal Cancer

Variables	Total no.	CAC No.	CAC (%)	ACM No.	ACM (%)	MAC No.	MAC (%)	*P* value[a]	*P* value[b]	*P* value[c]
Total No.	425	371		38		16				
Age (years)										
Mean ± SD	64.1 ± 10.1	63.2 ± 11.7		63.2 ± 11.7		67.0 ± 10.7		NS (0.611)*	NS (0.255)*	NS (0.275)*
<60	129	114	(30.7)	13	(34.2)	2	(12.5)	NS (0.713)	NS (0.164)	NS (0.182)
≥60	296	257	(69.3)	25	(65.8)	14	(87.5)			
Gender								NS (>0.999)	0.018	NS (0.056)
Male	250	214	(57.7)	22	(57.9)	14	(87.5)			
Female	175	157	(42.3)	16	(42.1)	2	(12.5)			
Tumor location								NS (0.087)[d]	NS (0.531)[d]	NS (0.728)[d]
Proximal	109	90	(24.3)	14	(36.8)	5	(31.3)			
Distal	181	163	(43.9)	12	(31.6)	6	(37.5)			
Rectum	135	118	(31.8)	12	(31.6)	5	(31.3)			
Intramural vascular invasion								NS (0.504)	NS (0.172)	NS (0.493)
Negative	76	63	(17.0)	8	(21.1)	5	(31.3)			
Positive	349	308	(83.0)	30	(78.9)	11	(68.8)			
pT stage								NS (0.989)**	0.023**	NS (0.052)**
pT1 or pT2	84	76	(20.5)	8	(21.1)	0	(0.0)			
pT3	285	248	(66.8)	25	(65.8)	12	(75.0)			
pT4	56	47	(12.7)	5	(13.2)	4	(25.0)			
pN stage								NS (0.844)	NS (0.560)	NS (0.746)
pN1	317	279	(75.2)	28	(73.7)	11	(68.8)			
pN2	108	92	(24.8)	10	(26.3)	5	(31.3)			
Stage (7th AJCC)								NS (0.761)**	NS (0.166)**	NS (0.158)**
IIIA	79	71	(19.1)	8	(21.1)	0	(0.0)			
IIIB	274	237	(63.9)	24	(63.2)	13	(81.3)			
IIIC	72	63	(17.0)	6	(15.8)	3	(18.8)			
KRAS gene status								NS (0.218)	NS (0.766)	NS (0.728)
Missing	136	115		19		2				
Wild type	180	163	(63.7)	9	(47.4)	8	(57.1)			
Mutant	109	93	(36.3)	10	(52.6)	6	(42.9)			
BRAF gene status								NS (0.066)	0.025	NS (0.415)
Missing	19	15		3		1				
Wild type	384	342	(96.1)	31	(88.6)	12	(80.0)			
Mutant	21	14	(3.9)	4	(11.4)	3	(20.0)			
Preoperative CEA (ng/ml)								NS (0.101)	NS (0.571)	NS (>0.999)
Missing	2	1		0		1				
<5	287	255	(68.9)	21	(55.3)	9	(60.0)			
≥5	136	115	(31.1)	17	(44.7)	6	(40.0)			
Preoperative CA19-9 (ng/ml)								NS (0.094)	NS (0.445)	NS (>0.999)
Missing	2	1		0		1				
<37	361	320	(86.5)	29	(76.3)	12	(80.0)			
≥37	62	50	(13.5)	9	(23.7)	3	(20.0)			
LNR								NS (0.297)	NS (>0.999)	NS (0.709)
<20	312	327	(88.8)	31	(82.4)	14	(86.7)			
≥20	113	44	(11.2)	7	(17.6)	2	(13.3)			
Adjuvant chemotherapy								NS (>0.999)	NS (0.513)	NS (0.713)
Absence	80	69	(18.1)	7	(18.4)	4	(25.0)			
Presence	345	306	(81.9)	31	(81.6)	12	(75.0)			

AJCC, 7th edition of the American Joint Committee on Cancer; Proximal, cecum to transverse colon; Distal, splenic flexure to sigmoid; LNR, lymph node ratio (ratio between metastatic and examined lymph nodes); MSS, microsatellite stable; MSI-H, microsatellite instability-high; *BRAF*-W, *BRAF*-wild type; *BRAF*-M, BRAF-mutation; CAC, conventional adenocarcinoma, ACM, adenocarcinoma with mucinous component; MAC, mucinous adenocarcinoma; NS, not significant.

[a]*P* value between CAC vs ACM.

[b]*P* value between CAC vs MAC.

[c]*P* value between ACM vs MAC.

[d]*P* value between proximal vs distal colon.

*unpaired Student *t* test.

**Mann-Whitney U-test; the remainig variables, Fisher's exact test.

In prognostic analysis for DFS, MAC resulted in significantly worse outcomes than CAC or ACM patients (Fig. 2) and was an independent prognostic factor in multivariate prognostic analysis (Table II), a finding consistent with previous studies [14]. MAC was also independently associated with OS, indicating one of the most aggressive phenotypes in stage III CRCs. Unexpectedly, ACM had unfavorable outcomes regarding OS, despite having a similar DFS to that of CAC (Figs. 2 and 3A). To resolve this discrepancy, as DFS is an excellent predictor of OS in stage III CRC [23], we examined OS defined as time from tumor recurrence to death in 118 patients with recurrence. We observed that patients with ACM had similar outcomes to those with MAC after recurrence (Fig. 3B). In addition, ACM exhibited similar molecular

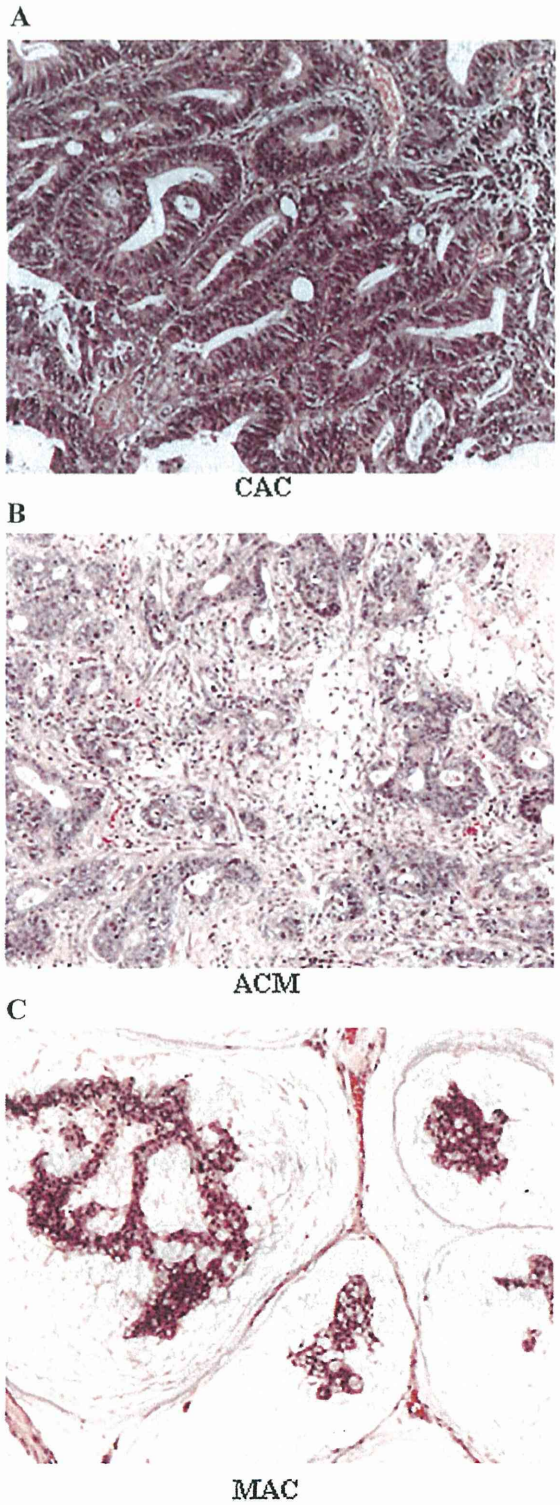

Fig. 1. Representative images of mucinous histology. (A) conventional adenocarcinoma (CAC); (B) adenocarcinoma with mucinous component (ACM); (C) mucinous adenocarcinoma (MAC). Slides were counterstained with hematoxylin and eosin. Original magnification, ×100.

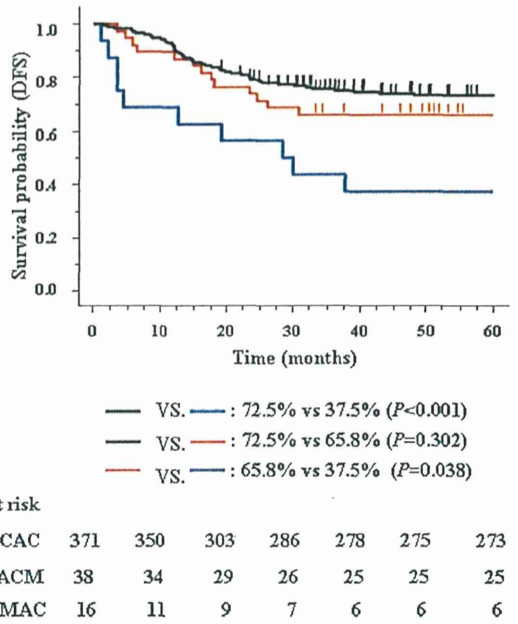

Fig. 2. Kaplan-Meier curves of 5-year DFS according to mucinous histology in 425 stage III CRC patients. Log-rank test was used to compare distribution among mucinous histology. CAC, conventional adenocarcinoma; ACM, adenocarcinoma with mucinous component; MAC, mucinous adenocarcinoma.

features to those of MAC, such as *BRAF* mutation and TP53 alteration [12]. Taken together, these findings suggest that MAC may consist of a higher proportion of cancer cells with the potential to execute multiple steps of the invasion-metastasis cascade than ACM, leading to an increased risk of metastasis in proportion to the number of potent cells already launched from the primary tumor before surgery and thereby significant difference in DFS between MAC and ACM. Once colonized at distant sites, MAC and ACM exhibit a similarly aggressive phenotype in the tumor-environment formed by potent cells with similar molecular features, leading to the similar OS.

Mucins are major glycoproteins of the gastrointestinal tract with two structurally and functionally distinct classes: secreted gel-forming mucins, such as MUC2; and transmembrane mucins, such as MUC1 [24]. MUC2 can contribute to the suppression of carcinogenesis [25], and its expression is decreased in CAC but not in MAC [26]. In contrast, MUC2 may play a role in the metastasis of MAC [24], and frequent MUC1 expression is known to lead to the aggressive behavior of MAC [26,27]. Thus mucins clearly exhibit differing detrimental roles and distributions in MAC and CAC. While an increased volume of mucins may promote physical translocation [28], the volume of metastatic tumors is equal to or lower than that of the primary tumor of MAC (Supplementary Table III), in line with previous studies [7,12], suggesting that the quantity of mucins does not necessarily reflect the aggressive phenotype, which appears to be reflected by cancer cells producing crucial quality of mucins such as MUC1. In addition, MAC shares similar mutational patterns and unfavorable outcomes, irrespective of organ sites, such as ovary or colorectum [7]. Identifying the detailed mechanism is a promising avenue for developing therapeutic approaches tailored to mucinous histology.

In the present study, DFS and OS were relatively favorable, compared to the previous pivotal studies [4,5]. One the other hand, in two recent phase III studies for non-inferiority between the 5-FU class in