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Journal

Journal of Clinical Oncology, 31(29)

ISSN

0732-183X

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Publication Date

2013-10-10

DOI

10.1200/jco.2013.48.9591

Peer reviewed

VOLUME 31 · NUMBER 29 · OCTOBER 10 2013

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Prognostic Impact of Deficient DNA Mismatch Repair in Patients With Stage III Colon Cancer From a Randomized Trial of FOLFOX-Based Adjuvant Chemotherapy

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Published online ahead of print at www.jco.org on September 9, 2013.

Written on behalf of the Alliance for Clinical Trials in Oncology.

Supported by a National Cancer Institute Senior Scientist Award (Grant No. K05CA-142885 to F.A.S) and the North Central Cancer Treatment Group Biospecimen Resource National Institutes of Health Grant No. CA-114740. Support for correlative studies was also provided by unrestricted funds from Bristol-Myers Squibb, ImClone Systems, sanofi-aventis, and Pfizer.

Presented in part at the European Society for Medical Oncology 14th World Congress on Gastrointestinal Cancer, June 27-30, 2012, Barcelona, Spain.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/13/3129w-3664w/$20.00

DOI: 10.1200/JCO.2013.48.9591

ABSTRACT

Purpose
The association of deficient DNA mismatch repair (dMMR) with prognosis in patients with colon cancer treated with adjuvant fluorouracil, leucovorin, and oxaliplatin (FOLFOX) chemotherapy remains unknown.

Patients and Methods
Resected, stage III colon carcinomas from patients (N = 2,686) randomly assigned to FOLFOX ± cetuximab (North Central Cancer Treatment Group N0147 trial) were analyzed for mismatch repair (MMR) protein expression and mutations in *BRAF*^{V600E} (exon 15) and *KRAS* (codons 12 and 13). Association of biomarkers with disease-free survival (DFS) was determined using Cox models. A validation cohort (Cancer and Leukemia Group B 88903 trial) was used.

Results
dMMR was detected in 314 (12%) of 2,580 tumors, of which 49.3% and 10.6% had *BRAF*^{V600E} or *KRAS* mutations, respectively. MMR status was not prognostic overall (adjusted hazard ratio [HR], 0.82; 95% CI, 0.64 to 1.07; $P = .14$), yet significant interactions were found between MMR and primary tumor site ($P_{interaction} = .009$) and lymph node category (N1 *v* N2; $P_{interaction} = .014$). Favorable DFS was observed for dMMR versus proficient MMR proximal tumors (HR, 0.71; 95% CI, 0.53 to 0.94; $P = .018$) but not dMMR distal tumors (HR, 1.71; 95% CI, 0.99 to 2.95; $P = .056$), adjusting for mutations and covariates. Any survival benefit of dMMR was lost in N2 tumors. Mutations in *BRAF*^{V600E} (HR, 1.37; 95% CI, 1.08 to 1.70; $P = .009$) or *KRAS* (HR, 1.44; 95% CI, 1.21 to 1.70; $P < .001$) were independently associated with worse DFS. The observed MMR by tumor site interaction was validated in an independent cohort of stage III colon cancers ($P_{interaction} = .037$).

Conclusion
The prognostic impact of MMR depended on tumor site, and this interaction was validated in an independent cohort. Among dMMR cancers, proximal tumors had favorable outcome, whereas distal or N2 tumors had poor outcome. *BRAF* or *KRAS* mutations were independently associated with adverse outcome.

J Clin Oncol 31:3664-3672. © 2013 by American Society of Clinical Oncology

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and is a leading cause of cancer death worldwide.[1] The majority of newly diagnosed patients present with local or regional disease[2] and can potentially be cured by a combination of surgery and chemotherapy. However, differences in clinical outcomes exist that depend on tumor biology. CRCs can be divided into those with microsatellite instability (MSI) and those that are microsatellite stable but show chromosomal instability. MSI is a consequence of deficient DNA mismatch repair (dMMR)[3,4] that results in an accumulation of errors within microsatellite regions producing high mutation rates. Most MSI/dMMR CRCs are sporadic and are associated with the CpG island methylator phenotype (CIMP)[5,6] and have frequent *BRAF*^{V600E} mutations.[7] The *BRAF* oncogene encodes a serine/threonine kinase and is a downstream effector of the Ras/Raf/MAPK signaling pathway.[8,9] *BRAF*^{V600E} or *KRAS* mutations predict nonresponse to anti–epidermal growth factor receptor antibody therapy in metastatic CRCs, although only *KRAS* has been validated.[10] In metastatic CRCs, *BRAF*^{V600E} mutations[11] have been associated with adverse clinical outcome.[12]

CRCs with MSI/dMMR have distinct clinicopathologic features including a propensity for the proximal colon.[13,14] Most studies have shown that MSI/dMMR is associated with a favorable prognosis in patients with CRC.[14-17] An important limitation of these studies is that patients were not treated with the current standard adjuvant chemotherapy regimen of fluorouracil (FU), leucovorin, and oxaliplatin (FOLFOX).[18] This issue is complex in that MSI/dMMR is associated with resistance to FU in vitro[19] and in vivo,[20,21] whereas in vitro chemotherapy sensitivity to oxaliplatin seems to be independent of the mismatch repair (MMR) system.[22]

We determined the association of MMR status with disease-free survival (DFS) in prospectively collected, stage III colon carcinomas from patients treated in a phase III trial of FOLFOX alone or combined with cetuximab as adjuvant chemotherapy (North Central Cancer Treatment Group N0147 trial).[23] In this trial, the addition of cetuximab to FOLFOX failed to improve DFS overall or in patients with wild-type *KRAS* tumors.[23] We examined the prognostic impact of MMR on DFS adjusting for the mutation status of $BRAF^{V600E}$ or *KRAS* genes. Given that dMMR tumors are located primarily in the proximal colon and show a reduced propensity for metastases,[24-26] we also examined the prognostic impact of MMR stratified by tumor site and lymph node category (N1 *v* N2). An independent cohort of patients with stage III colon cancer from another phase III adjuvant study was used for validation.[27]

PATIENTS AND METHODS

Study Population

Patients (N = 2,686) with resected, stage III (any T, N1 or N2, M0) adenocarcinoma of the colon participated in a phase III randomized trial of modified FOLFOX6 (n = 1,337) or modified FOLFOX6 + cetuximab (n = 1,349).[23] The trial was modified after initiation to restrict random assignment to patients whose tumors expressed wild-type *KRAS*.[23] Biospecimens were prospectively collected and required for study participation. A central pathology review was performed. Patients received chemotherapy within 10 weeks of surgery. Stratification factors included the following: number of metastatic lymph nodes (one to three *v* ≥ four nodes), histologic grade (high [poorly differentiated or undifferentiated] *v* low [well or moderately differentiated]), and T stage (T1-2 *v* T3 *v* T4). Proximal tumor sites included cecum and ascending and transverse colon; distal sites included splenic flexure and descending and sigmoid colon. The study was approved by the Mayo Clinic Institutional Review Board and the North Central Cancer Treatment Group (now part of Alliance for Clinical Trials in Oncology).

DNA MMR Proteins

MMR protein (MLH1, MSH2, and MSH6) expression was analyzed in formalin-fixed, paraffin-embedded tumor sections using an immunoperoxidase method.[28] Monoclonal antibodies included mouse antihuman MLH1 (clone G168-15; Biocare Medical, Concord, CA), antihuman MSH2 (clone FE11; Biocare Medical), and antihuman MSH6 (clone BC/44; Biocare Medical). MMR protein loss was defined as the absence of nuclear staining in tumor cells in the presence of positive nuclear staining in normal colonic epithelium and lymphocytes. Expression was scored by one GI pathologist (T.C.S.). Tumors were classified as dMMR (*v* proficient MMR [pMMR]) if loss of one or more MMR proteins was detected.

BRAF and KRAS Gene Mutations

Mutation status was determined using genomic DNA extracted from macrodissected tumor tissue. Testing for the $BRAF^{V600E}$ hotspot mutation in exon 15 was performed using a multiplex allele-specific, real-time polymerase chain reaction–based assay and an automated sequencing technique.[29] Primer sequences included the following: wild-type forward, NED-TGATTTTGGTCATGCTACAGT; mutant forward, 6-Fam-CAGTGATTTTGCTCTAGCTTCAGA; and reverse, GTTTCTTTCTAGTAACTCAGCAGC. *KRAS* mutation status was analyzed in extracted DNA using the DxS mutation test kit KR-03/04 (DxS, Manchester, United Kingdom), assessing for seven different mutations in codons 12 and 13.[30] For both genes, mutational analysis was performed in a Clinical Laboratory Improvement Amendments–compliant laboratory at the Mayo Clinic. All biomarker data were analyzed with investigators blinded to patient outcomes.

Validation Cohort

The validation cohort consisted of patients with stage III colon cancer (N = 1,264) randomly assigned to receive FU plus leucovorin or FU, leucovorin, and irinotecan as adjuvant chemotherapy (Cancer and Leukemia Group B [CALGB] 89803 trial).[27] No differences in survival were observed between the treatment arms. MMR status was retrospectively determined in available tissues (n = 891) by analysis of MLH1 and MSH2 protein expression and/or by MSI, as previously described.[27] The mutation status of *KRAS* and $BRAF^{V600E}$ was previously analyzed in a subset (n = 571) of the cohort.[31,32]

Statistical Analysis

Analysis of the primary study end point of DFS was previously reported for the treatment arms.[23] All patients were censored for DFS at 5 years after random assignment; intent-to-treat principles were used. The two treatment arms were pooled given the lack of statistically significant differences in DFS rates and the lack of significant interactions between any of the biomarkers and treatment. Median follow-up time for 2,213 surviving patients was 4.1 years (range, 0.0 to 7.5 years). Kaplan-Meier methods were used to describe the distributions of DFS.[33] Univariate Cox proportional hazards models[34] were used to explore the associations of patient characteristics and biomarkers with outcome. Thereafter, multivariable models were used, and unless otherwise specified, all models were adjusted for stratification factors (T stage, nodal category, and grade), primary tumor site, age, sex, treatment, and status of MMR, $BRAF^{V600E}$, or *KRAS*. Interactions between biomarkers or biomarkers with treatment were assessed. Analyses were reported using a validation cohort and to evaluate the robustness of the results. Two-sided *P* values are reported; $P < .05$ was considered statistically significant. Analyses were performed using SAS version 9.2 (SAS Institute, Cary NC) and R version 2.14.[35]

RESULTS

Study Population and Biomarkers

Characteristics of the patient population with stage III colon carcinomas (N = 2,686) are listed in Table 1 stratified by MMR, $BRAF^{V600E}$, or *KRAS* status. In the full cohort, dMMR was detected in 314 (12%) of 2,580 tumors, and mutations were present in $BRAF^{V600E}$ or *KRAS* in 14% (346 of 2,515 tumors) and 28% (716 of 2,579 tumors), respectively (Table 1). Of note, our clinical trial population was enriched with wild-type *KRAS* tumors based on study eligibility criteria.[23] $BRAF^{V600E}$ and *KRAS* mutations were mutually exclusive.

The number of cancers located in the proximal or distal colon was equal in the study population. Patients with proximal versus distal tumors were significantly older (median age, 61 *v* 56 years, respectively; $P < .001$) and more often women, and their tumors were more likely to have high histologic grade and increased T stage (T4 *v* T3 *v* T1-2; all $P < .02$; Table 1). Furthermore, proximal versus distal cancers were significantly more likely to show dMMR (21% *v* 2.8%, respectively; $P < .001$) and mutations in $BRAF^{V600E}$ (23.3% *v* 4.3%, respectively; $P < .001$) or *KRAS* (32.8% *v* 22.6%, respectively; $P < .001$; Table 1).

DNA MMR Status

dMMR was detected in 12% of tumors that exhibited loss of MLH1 (n = 264; 84%), MSH2 (n = 46), or MSH6 (n = 53) protein

Table 1. Clinicopathologic Variables Are Stratified by Mismatch Repair, *BRAF*^{V600E}, *KRAS*, and Primary Tumor Site

	Mismatch Repair					*BRAF*^{V600E}					*KRAS*					Tumor Site				
	dMMR (n = 314)		pMMR (n = 2,266)			Mutant (n = 346)		Wild Type (n = 2,169)			Mutant (n = 716)		Wild Type (n = 1,863)			Proximal (n = 1,326)		Distal (n = 1,323)		
Variable	No.	%	No.	%	*P*	No.	%	No.	%	*P*	No.	%	No.	%	*P*	No.	%	No.	%	*P*
Age, years					< .001*					< .0011					.5035*					< .001*
Median	62.0		58.0			65.0		57.0			59.0		58.0			61.0		56.0		
Range	28.0-86.0		19.0-85.0			31.0-86.0		19.0-85.0			22.0-85.0		19.0-86.0			19.0-86.0		19.0-85.0		
Study arm					.0805†					.0533†					.1174†					.0038†
Modified FOLFOX6	142	45.2	1,144	50.5		155	44.8	1,093	50.4		374	52.2	909	48.8		623	47.0	696	52.6	
Modified FOLFOX6 + cetuximab	172	54.8	1,122	49.5		191	55.2	1,076	49.6		342	47.8	954	51.2		703	53.0	627	47.4	
Sex					< .001†					< .001†					.3199†					.0166†
Female	185	58.9	1,038	45.8		224	64.7	968	44.6		350	48.9	870	46.7		663	50.0	600	45.4	
Male	129	41.1	1,228	54.2		122	35.3	1,201	55.4		366	51.1	993	53.3		663	50.0	723	54.6	
Histologic grade‡					< .001†					< .001†					.0021†					< .001†
High	166	52.9	494	21.8		167	48.3	483	22.3		153	21.4	508	27.3		437	33.0	239	18.1	
Low	148	47.1	1,772	78.2		179	51.7	1,686	77.7		563	78.6	1,355	72.7		889	67.0	1,084	81.9	
Lymph node status‡					.6186†					< .001†					.0191†					.2417†
N1: 1-3 nodes	187	59.6	1,316	58.1		170	49.1	1,295	59.7		443	61.9	1,058	56.8		754	56.9	782	59.1	
N2: ≥ 4 nodes	127	40.4	950	41.9		176	50.9	874	40.3		273	38.1	805	43.2		572	43.1	541	40.9	
T stage‡					.0076†					.0017†					.9078†					< .001†
Missing	0		1			0		1			1		0			0		1		
T1 or T2	30	9.6	358	15.8		37	10.7	338	15.6		106	14.8	278	14.9		160	12.1	241	18.2	
T3	240	76.4	1,658	73.2		253	73.1	1,599	73.8		524	73.3	1,375	73.8		993	74.9	952	72.0	
T4	44	14.0	249	11.0		56	16.2	231	10.7		85	11.9	210	11.3		173	13.0	129	9.8	
Tumor site					< .001†					< .001†					< .001†					
Missing	7		28			5		32			12		25							
Proximal	271	88.3	1,001	44.7		287	84.2	944	44.2		416	59.1	852	46.4						
Distal	36	11.7	1,237	55.3		54	15.8	1,193	55.8		288	40.9	986	53.6						
KRAS					< .001†					< .001†										< .001†
Missing	4		36			2		3								58		49		
Mutant	33	10.6	676	30.3		0	0.0	687	31.7							416	32.8	288	22.6	
Wild type	277	89.4	1,554	69.7		344	100.0	1,479	68.3							852	67.2	986	77.4	
BRAF^{V600E}					< .001†										< .001†					< .001†
Missing	10		90								29		40			95		76		
Mutant	150	49.3	190	8.7							0	0.0	344	18.9		287	23.3	54	4.3	
Wild type	154	50.7	1,986	91.3							687	100.0	1,479	81.1		944	76.7	1,193	95.7	
Mismatch repair										< .001†					< .001†					< .001†
Missing						6		29			7		32			54		50		
pMMR						190	55.9	1,986	92.8		676	95.3	1,554	84.9		1,001	78.7	1,237	97.2	
dMMR						150	44.1	154	7.2		33	4.7	277	15.1		271	21.3	36	2.8	

Abbreviations: dMMR, deficient mismatch repair; FOLFOX6, fluorouracil, leucovorin, and oxaliplatin[23]; pMMR, proficient mismatch repair.
*Kruskal-Wallis test.
†χ^2 test.
‡Stratification factor.

expression by immunohistochemistry. Three tumors with MLH1 loss also lacked MSH6. Consistent with prior studies,[14-17,24] dMMR was significantly associated with older age, female sex, proximal site, high-grade histology, and higher T stage (all $P < .008$; Table 1). The proportion of patients with N2 nodal classification ($\geq$ four positive lymph nodes)[36] did not differ by MMR status (dMMR *v* pMMR: 127 [40%] *v* 950 [42%], respectively). Among dMMR tumors, 150 (49.3%) carried *BRAF*^{V600E} mutations and 33 (10.6%) had *KRAS* mutations (Table 1).

BRAF^{V600E} and KRAS Mutational Status

As with dMMR, mutated *BRAF*^{V600E} was significantly associated with older age, female sex, proximal site, high-grade histology, and higher T stage (all $P < .002$; Table 1). In contrast to dMMR, *BRAF*^{V600E} mutations were significantly more frequent in N2 versus N1 tumors ($P < .001$). *KRAS* mutations were detected in only 4.7% of dMMR tumors ($P < .001$) and were significantly associated with proximal site, low-grade histology, and N1 stage (all $P < .02$; Table 1).

Prognostic Impact of Clinicopathologic Variables and Biomarkers

Proximal tumor site and higher T or N stage were each significantly associated with inferior DFS and were independent of other covariates (Table 2). For the biomarker analyses, we combined patients from both treatment arms because we did not find any statistically significant interactions between MMR, *BRAF*^{V600E}, or *KRAS* status and cetuximab treatment. In the full cohort, MMR status was not significantly associated with DFS by univariate analysis (hazard ratio [HR], 1.04; 95% CI, 0.83 to 1.29; $P = .7547$; Table 2) or after adjustment for clinical variables, *BRAF*^{V600E}, or *KRAS* status (HR, 0.82; 95% CI, 0.64 to 1.07; $P = .14$; Fig 1). Mutated versus wild-type *BRAF*^{V600E} and *KRAS* were each significantly associated with worse DFS in univariate and multivariate analyses (Table 2; Fig 1).

Analysis of MMR status by tumor site revealed that dMMR versus pMMR tumors in the proximal colon were associated with better DFS (HR, 0.78; 95% CI, 0.61 to 1.00; $P = .053$); this association was statistically significant after adjustment for *BRAF*^{V600E}, *KRAS*, and clinicopathologic variables (HR, 0.71; 95% CI, 0.53 to 0.94; $P = .018$; Fig 2). In contrast, dMMR versus pMMR tumors in the distal colon were associated with significantly worse DFS by univariate analysis (HR, 1.91; 95% CI, 1.11 to 3.26; $P = .018$); this association was marginally significant in a multivariable analysis (HR, 1.71; 95% CI, 0.99 to 2.95; $P = .056$; Fig 2). Consistent with these findings, we found a statistically significant interaction between MMR status and primary tumor site ($P_{\text{interaction}} = .0089$; Table 2). Within MMR categories, pMMR tumors of the proximal versus distal colon were associated with a significantly worse DFS (adjusted HR, 1.27; 95% CI, 1.08 to 1.52; $P = .0047$).

Among patients with dMMR, those with N2 versus N1 disease had significantly worse DFS (HR, 3.24; 95% CI, 2.12 to 4.95; $P < .001$; adjusted HR, 3.49; 95% CI, 2.19 to 5.54; $P < .001$; Fig 3). A similar yet weaker association for the effect of N stage on DFS was observed among pMMR tumors (HR, 2.26; 95% CI, 1.93 to 2.63; $P < .001$; adjusted HR, 2.05; 95% CI, 1.74 to 2.41; $P < .001$; Fig 3). Further analysis revealed a statistically significant interaction between MMR status and lymph node category ($P_{\text{interaction}} = .0135$).

Among patients with dMMR tumors, those with mutated versus wild-type *BRAF* had similar DFS rates (HR, 1.33; 95% CI, 0.87 to 2.04;

Table 2. Univariate and Multivariate Cox Proportional Hazards Regression Models for Disease-Free Survival

Variable	No. of Patients	No. of Events	HR	95% CI	P*
Univariate analysis					
T stage (reference: T1/T2)	2,685	773			
T3			2.75	2.06 to 3.69	< .001
T4			4.97	3.59 to 6.89	< .001
Histologic grade: high *v* low	2,686	773	1.37	1.17 to 1.60	< .001
Lymph node status: N2 *v* N1	2,686	773	2.35	2.04 to 2.72	< .001
Tumor site: distal *v* proximal	2,649	764	0.70	0.61 to 0.81	< .001
Study arm: mFOLFOX6 +cetuximab *v* mFOLFOX6	2,686	773	1.06	0.92 to 1.22	.4010
Sex: male *v* female	2,686	773	1.13	0.98 to 1.31	.0802
Age (per year)	2,686	773	1.01	1.00 to 1.01	.0294
KRAS: mutant *v* wild type	2,579	749	1.34	1.15 to 1.55	< .001
BRAF^{V600E}: mutant *v* wild type	2,515	731	1.34	1.11 to 1.63	.0028
MMR: dMMR *v* pMMR	2,580	752	1.04	0.83 to 1.29	.7536
MMR/*BRAF* (reference: pMMR and wild-type *BRAF*)	2,480	724			
dMMR and mutant *BRAF*^{V600E}			1.17	0.87 to 1.57	.2958
dMMR and wild-type *BRAF*			0.89	0.64 to 1.23	.4742
pMMR and mutant *BRAF*^{V600E}			1.50	1.18 to 1.91	< .001
BRAF/*KRAS* (reference: wild-type *BRAF* and wild-type *KRAS*)	2,510	729			
Mutant *BRAF*^{V600E} and wild-type *KRAS*			1.52	1.24 to 1.87	< .001
Wild-type *BRAF* and mutant *KRAS*			1.47	1.25 to 1.73	< .001
Multivariate analysis					
T stage (reference: T1/T2)					
T3			2.26	1.66 to 3.07	< .001
T4			4.08	2.90 to 5.76	< .001
Histologic grade: high *v* low			1.14	0.97 to 1.35	.1133
Nodal status: N2 *v* N1			2.17	1.86 to 2.52	< .001
Study arm: modified FOLFOX6 + cetuximab *v* modified FOLFOX6			1.07	0.93 to 1.24	.3559
Sex: male *v* female			1.16	1.00 to 1.35	.0530
Age (per year)			1.00	1.00 to 1.01	.4146
KRAS: mutant *v* wild type			1.44	1.21 to 1.70	< .001
BRAF^{V600E}: mutant *v* wild type			1.37	1.08 to 1.74	.009
MMR/site (reference: pMMR and proximal)					.0089†
dMMR and distal			1.28	0.75 to 2.20	
dMMR and proximal			0.73	0.55 to 0.96	
pMMR and distal			0.79	0.67 to 0.93	

Abbreviations: dMMR, deficient mismatch repair; FOLFOX6, fluorouracil, leucovorin, and oxaliplatin; HR, hazard ratio; MMR, mismatch repair; pMMR, proficient mismatch repair.

*χ^2 test.

†*P* value for the test of the interaction of MMR and tumor site includes all of the covariates shown (ie, *KRAS*, *BRAF*^{V600E}, T stage, histologic grade, No. of positive nodes, age, arm, sex, and MMR $\times$ site).

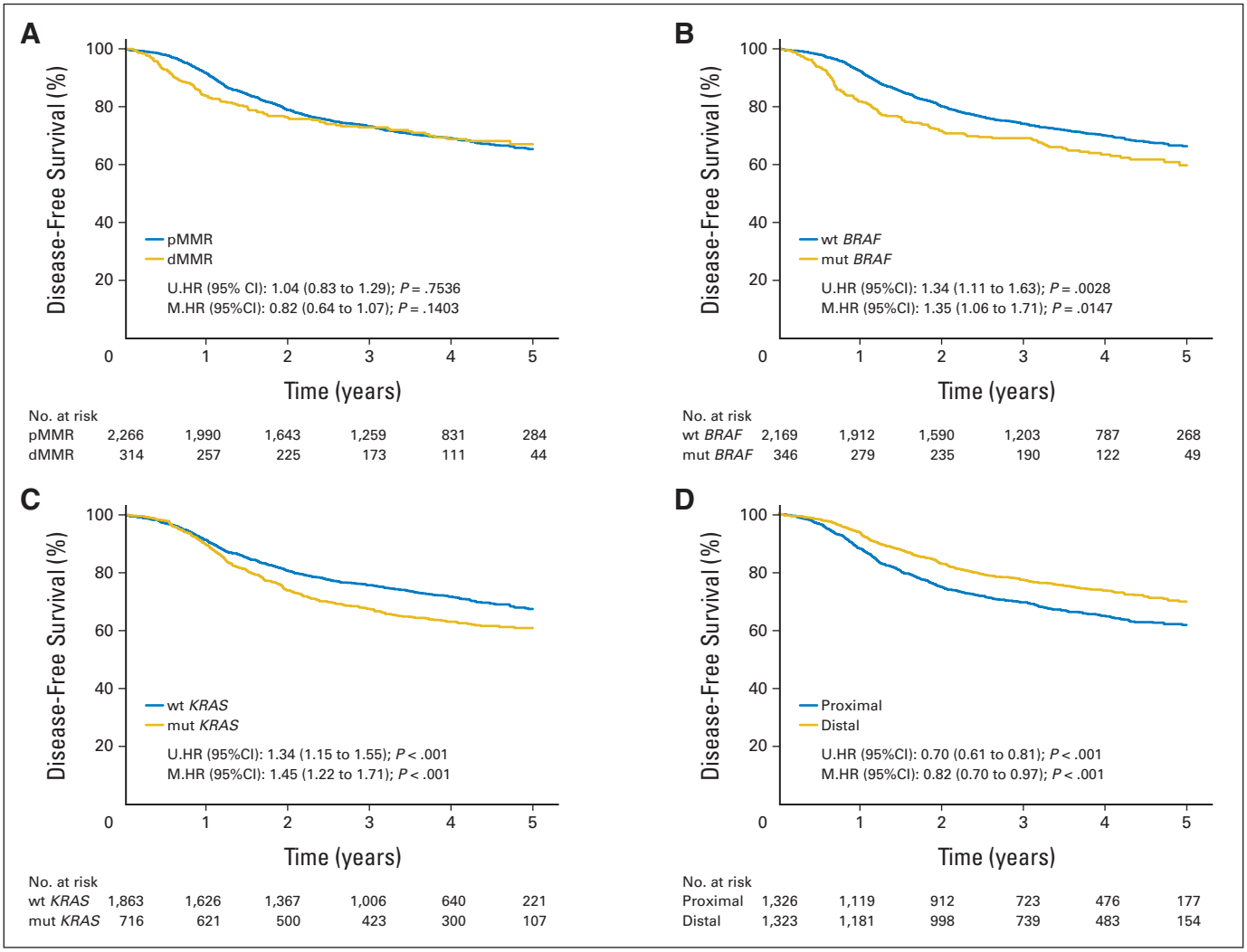

Fig 1. Association of (A) DNA mismatch repair (MMR) status, mutations in (B) *BRAF*^{V600E} or (C) *KRAS*, and (D) primary tumor site with disease-free survival in stage III colon carcinomas. Cox models are adjusted for MMR, *KRAS*, *BRAF*^{V600E}, T stage, histologic grade, lymph node category, age, sex, treatment, and tumor site. dMMR, deficient DNA mismatch repair; HR, hazard ratio; M, multivariate; mut, mutant; pMMR, proficient DNA mismatch repair; U, univariate; wt, wild type.

$P = .19$; adjusted HR, 1.58; 95% CI, 0.88 to 2.82; $P = .12$; Fig 4). A similar outcome was also seen for dMMR tumors with mutated versus wild-type *KRAS* (Fig 4). Among patients with pMMR tumors, mutated *BRAF*^{V600E} was associated with significantly worse DFS (HR, 1.51; 95% CI, 1.19 to 1.92; $P < .001$; adjusted HR, 1.32; 95% CI, 1.01 to 1.73; $P = .044$) compared with wild-type cases (Fig 4). Similarly, pMMR tumors with mutated versus wild-type *KRAS* were associated with significantly poorer DFS (HR, 1.37; 95% CI, 1.17 to 1.60; $P < .001$; adjusted HR, 1.45, 95% CI, 1.22 to 1.73; $P < .001$; Fig 4). However, neither the MMR by *BRAF*^{V600E} (adjusted $P = .93$) nor the MMR by *KRAS* (adjusted $P = .38$) interaction tests were statistically significant. Compared with tumors with wild-type copies of both genes, those with mutation in either *BRAF*^{V600E} or *KRAS* showed significantly worse DFS (Table 2; Appendix Figure A1, online only).

Validation Cohort

We attempted to validate the interaction between MMR, tumor site, and nodal status in patients with stage III colon cancer from a phase III clinical trial of FU and leucovorin ± irinotecan where no difference in survival by adjuvant treatment arm was found (CALGB 89803 trial).[27] Consistent with findings in our study cohort, a statistically significant interaction was observed between MMR status and tumor site for DFS ($P_{\text{interaction}} = .037$), adjusted for clinical factors but not *BRAF*^{V600E} or *KRAS* status because mutation data were available in only 64% of patients in the validation cohort. The interaction for MMR and tumor site was similar and remained statistically significant when adjusting for an MMR and treatment arm interaction in addition to covariates. Among patients from the validation cohort with proximal cancers, dMMR was significantly associated with improved DFS (HR, 0.59; 95% CI, 0.41 to 0.86; $P = .0039$) after adjusting for N category, T stage, histologic grade, age, sex, and study arm. Among distal cancers, DFS did not differ significantly by MMR status (adjusted HR, 1.58; 95% CI, 0.72 to 3.46; $P = .2817$), although only 14 of 378 patients were in the distal dMMR subgroup. The statistically significant interaction between MMR and nodal status seen in our cohort was not confirmed in the validation cohort (adjusted $P = .7010$).

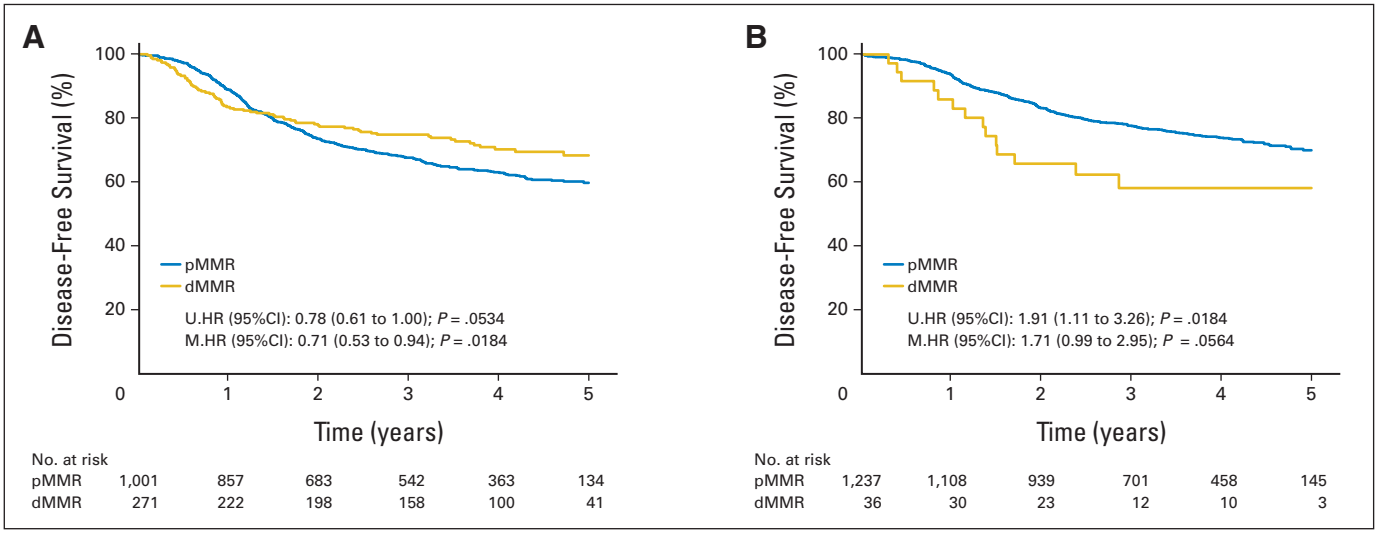

Fig 2. Impact of mismatch repair (MMR) status on disease-free survival by primary tumor site. Tumor site was categorized as (A) proximal versus (B) distal (see Patients and Methods). Cox models are adjusted for T stage, histologic grade, nodal category, age, sex, treatment, tumor site, and mutation status of *KRAS* and $BRAF^{V600E}$. dMMR, deficient DNA mismatch repair; HR, hazard ratio; M, multivariate; pMMR, proficient DNA mismatch repair; U, univariate.

DISCUSSION

We determined the prognostic impact of MMR status in prospectively collected stage III colon cancers from patients treated in an adjuvant study of FOLFOX ± cetuximab.[23] Study arms were combined for the analysis because the addition of cetuximab did not improve outcome and no interaction between treatment and any of the biomarkers was observed. Among 314 dMMR tumors (12%), 49% carried $BRAF^{V600E}$ mutations, and 10.6% had *KRAS* mutations. Although MMR status was not prognostic in the overall cohort, its association with DFS depended on tumor site as shown by a statistically significant interaction. Whereas dMMR versus pMMR was associated with a statistically significant DFS advantage in proximal tumors, dMMR was unexpectedly associated with worse DFS in distal tumors. Patients with distal (*v* proximal) dMMR tumors were significantly more likely to be younger (age 52 *v* 63 years, respectively; $P < .001$) and to have lower rates of mutation in $BRAF^{V600E}$ (20% *v* 54%, respectively; $P < .001$) but higher rates of mutation in *KRAS* (28% *v* 8%, respectively; $P < .001$). We validated our finding for the dependence of MMR on tumor site for DFS in an independent cohort of patients with stage III colon cancer randomly assigned to FU/leucovorin ± irinotecan in another adjuvant trial (CALGB 89803[27]). In that cohort, a statistically significant interaction was also found between MMR and tumor site for DFS. Taken together, these data indicate that the tumor site dependence of MMR for prognosis seems unrelated to the chemotherapy regimen used but is likely a result of intrinsic biologic factors.

We found a similar frequency of N2 disease in dMMR and pMMR tumors that was unexpected because dMMR has been

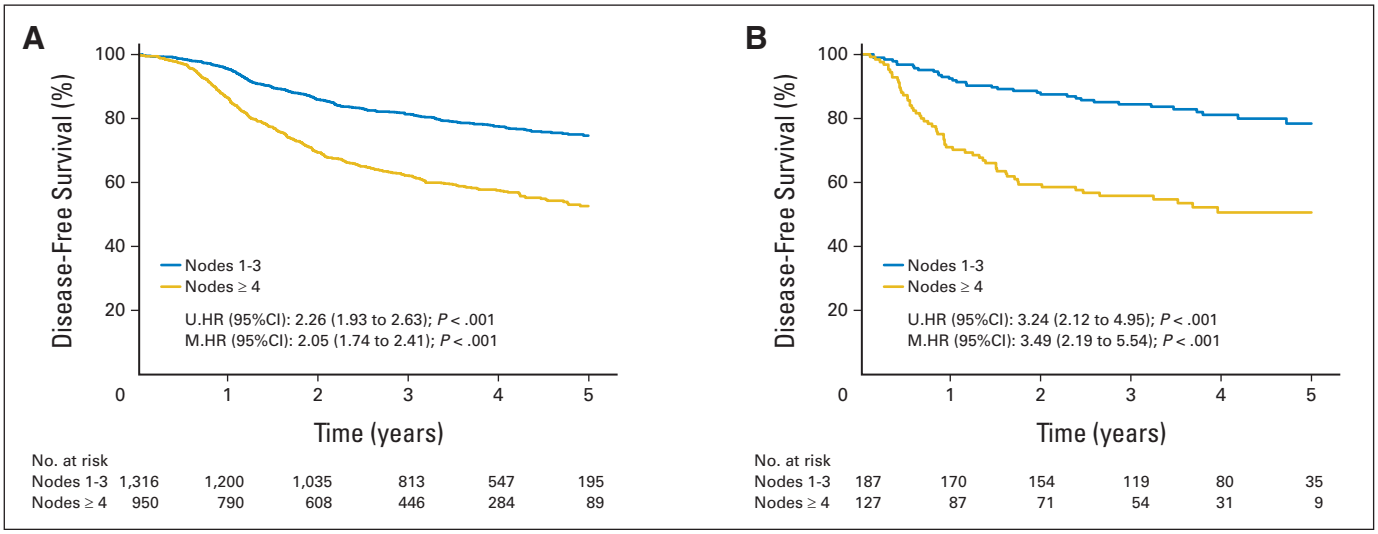

Fig 3. Analysis of the prognostic impact of lymph node category by mismatch repair (MMR) status on disease-free survival. Lymph node category includes N1 (one to three metastatic nodes) or N2 (≥ four metastatic nodes). MMR status is defined as (A) proficient MMR (pMMR) or (B) deficient MMR (dMMR). Cox models are adjusted for T stage, histologic grade, age, sex, treatment, tumor site, and mutational status of *KRAS* and $BRAF^{V600E}$. HR, hazard ratio; M, multivariate; U, univariate.

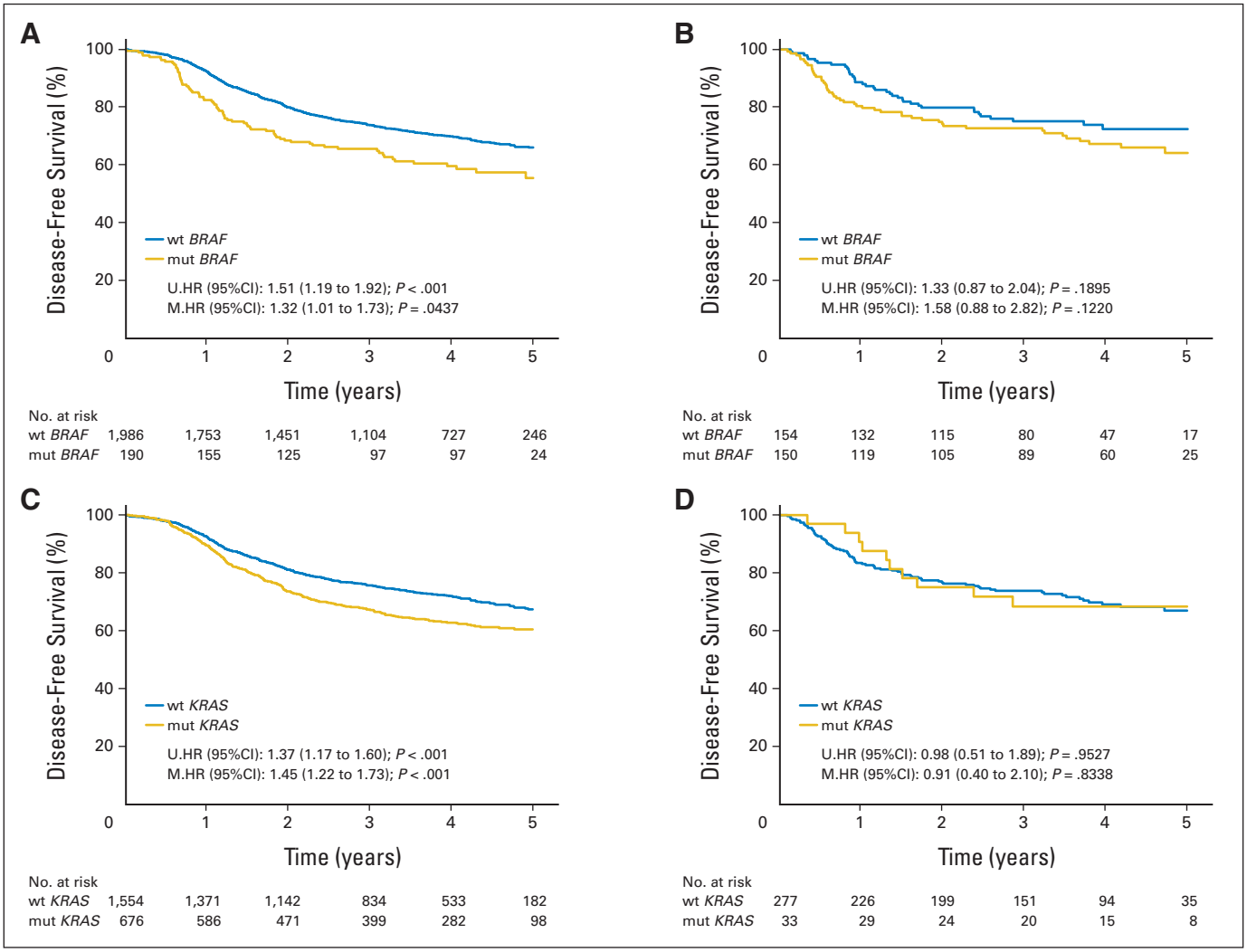

Fig 4. Impact of (A and B) $BRAF^{V600E}$ or (C and D) *KRAS* mutations on disease-free survival according to DNA mismatch repair (MMR) status. MMR status is defined as (A and C) proficient MMR (pMMR) or (B and D) deficient MMR (dMMR; see Patients and Methods). Cox models are adjusted for *KRAS*, $BRAF^{V600E}$, T stage, histologic grade, nodal category, age, sex, treatment, and tumor site. HR, hazard ratio; M, multivariate; mut, mutant; U, univariate; wt, wild type.

consistently associated with lower tumor stage at diagnosis and is uncommon in advanced CRCs.[16,24-26] In our cohort, a statistically significant interaction was found between MMR and nodal category for their impact on DFS. N2 versus N1 tumors showed significantly worse outcome in both dMMR and pMMR tumors, with a stronger effect in the former. This interaction, however, was not validated in the independent cohort. Although this may be related to an inability to adjust for $BRAF^{V600E}$ and *KRAS* status, which was not available in the full validation cohort,[31,32] we did validate the interaction between MMR and tumor site despite this limitation.

The lack of association of MMR status with DFS in the overall cohort deserves comment. Most prior studies examining the prognostic impact of MMR have not adjusted for $BRAF^{V600E}$ or *KRAS* status. Furthermore, we identified poor prognostic subgroups among dMMR tumors (ie, distal and N2 tumors). Although our cohort was restricted to stage III cancers, studies demonstrating a favorable outcome for dMMR versus pMMR colon cancers have generally combined stage II and III tumors,[14-17,25,27] suggesting that the favorable survival impact of dMMR may be stronger in earlier stage disease.[16,25,37] Another factor is treatment with the FOLFOX regimen because, in contrast with FU,[19] oxaliplatin chemotherapy sensitivity seems to be independent of the MMR system because oxaliplatin forms platinum adducts with DNA that cannot be repaired in MMR-deficient cells.[22] A study in stage II and III colon cancers (National Surgical Adjuvant Breast and Bowel Project C-07) found that the survival benefit of adding oxaliplatin to adjuvant FU/leucovorin was unrelated to MMR status.[38] Therefore, a survival benefit from oxaliplatin in both dMMR and pMMR tumors could attenuate any prognostic difference based on MMR status.

Although $BRAF^{V600E}$ mutations are strongly associated with dMMR, $BRAF^{V600E}$ mutations were significantly more frequent in N2 versus N1 cancers. In the overall cohort, $BRAF^{V600E}$ and *KRAS* mutations were each independently associated with a statistically significant reduction in DFS compared with wild-type tumors. When analyzed by MMR status, the prognostic impact of $BRAF^{V600E}$ or *KRAS* was limited to pMMR tumors, although the dMMR subgroups were admittedly smaller and the interaction test was not significant. Neither $BRAF^{V600E}$ nor *KRAS* mutations were associated with relapse-free survival in stage II or III colon cancers in the Pan-European Trials in

Adjuvant Colon Cancer-3,[25] National Surgical Adjuvant Breast and Bowel Project C-07/C-08,[38] or CALGB 89803[31,32] adjuvant studies; however, *BRAF*^{V600E} mutations were associated with shorter overall survival in these trials. In metastatic CRCs where dMMR is relatively uncommon, *BRAF*^{V600E} mutations have been associated with worse survival rates[12] (Medical Research Council FOCUS trial).[39]

In FOLFOX-treated stage III patients, our data indicate that any favorable impact of dMMR is restricted to proximal tumors or those that are N1. Dependence of the prognostic impact of MMR status on primary tumor site has implications for risk stratification and clinical decision making, particularly if our findings are found to apply to patients with stage II colon cancer. The potential for more selective use of adjuvant chemotherapy in patients with stage III disease does exist, as suggested by data from the Pan-European Trials in Adjuvant Colon Cancer-3 trial where a recursive partitioning analysis identified a tumor subgroup with dMMR and intact SMAD4 expression that had a clinical outcome similar to patients with stage II disease.[40,41] Strengths of our study include the prospective analysis of MMR and molecular markers using uniform assay methodology in a Clinical Laboratory Improvement Amendments–certified laboratory at a single tertiary medical center. We report the largest number of dMMR tumors from a single clinical trial. Study limitations include the limited median follow-up time of 4.1 years, although 3-year DFS has been shown to be a reliable surrogate for 5-year overall survival in adjuvant studies in colon cancer.[42]

In conclusion, the prognostic impact of dMMR on DFS was dependent on the primary tumor site in patients with stage III colon cancer, and this finding was validated in an independent cohort. Poor prognostic subgroups were observed within dMMR cancers that included distal site and N2 disease, which may have contributed to the nonsignificant overall impact of dMMR on DFS. Mutations in *BRAF*^{V600E} or *KRAS* were each independently associated with reduced DFS and may therefore provide clinically useful prognostic information in FOLFOX-treated patients.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: None **Consultant or Advisory Role:** Richard M. Goldberg, sanofi-aventis (U), Bayer (U), Jennerex (U) **Stock Ownership:** None **Honoraria:** Frank A. Sinicrope, Ventana Medical Systems; Richard M. Goldberg, Fresenius Kabi, Yakult **Research Funding:** Richard M. Goldberg, sanofi-aventis, Bayer **Expert Testimony:** None **Patents:** None **Other Remuneration:** None

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GLOSSARY TERMS

CIMP: The CpG island methylator phenotype is characterized by widespread, concordant promoter CpG island methylation resulting in silencing of many tumor suppressor genes. CIMP-high (high degree of CIMP) in colorectal cancer is associated with old age, female sex, proximal colon, BRAF mutation and MSI (microsatellite instability), and inversely with chromosomal instability, TP53 mutation, WNT/β-catenin (CTNNB1) activation, and genome-wide DNA hypomethylation.

DNA methylation: Methylation of bases contained in the DNA double helix, resulting in a loss of gene function. Generally occurring on cytosine residues in the DNA, methylation is important in regulating cell growth and differentiation and has resulted in the testing of DNA methyltransferase inhibitors as anticancer agents and differentiation agents.

Microsatellite instability: Microsatellites are repeating units in DNA of 1-5 basepairs that are ubiquitous, abundant, and repeated several times in eukaryotic genomes. The presence of microsatellites is associated with genomic instability, giving rise to mutations that involve the addition or subtraction of one or two repeat units.

Mismatch repair: One of four major pathways of DNA repair in mammalian cells. Mismatch repair recognizes and corrects errors in DNA replication leading to single base-pair mismatches or insertions/deletions in small repetitive tracts of DNA known as microsatellites.

Appendix

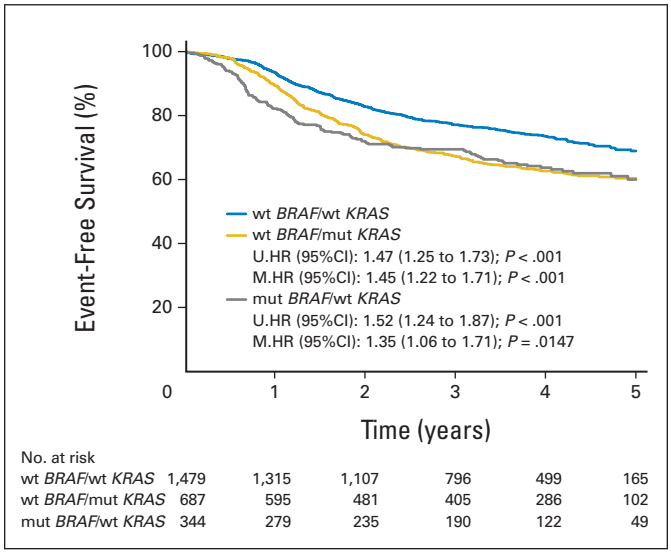

Fig A1. Association of *BRAF* ^{V600E} or *KRAS* mutation status as a combined variable with disease-free survival in stage III colon carcinomas. Cox models are adjusted for T stage, histologic grade, nodal category, age, sex, treatment, tumor site, and mismatch repair status. HR, hazard ratio; M, multivariate; mut, mutant; U, univariate; wt, wild type.