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Molecular Markers Identify Subtypes of Stage III Colon Cancer Associated With Patient Outcomes

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BACKGROUND & AIMS: Categorization of colon cancers into distinct subtypes using a combination of pathway-based biomarkers could provide insight into stage-independent variability in outcomes. **METHODS:** We used a polymerase chain reaction–based assay to detect mutations in *BRAF* (V600E) and in KRAS in 2720 stage III cancer samples, collected prospectively from patients participating in an adjuvant chemotherapy trial (NCCTG N0147). Tumors deficient or proficient in DNA mismatch repair (MMR) were identified based on detection of MLH1, MSH2, and MSH6 proteins and methylation of the *MLH1* promoter. Findings were validated using tumor samples from a separate set of patients with stage III cancer (n = 783). Association with 5-year disease-free survival was evaluated using Cox proportional hazards models. **RESULTS:** Tumors were categorized into 5 subtypes based on MMR status and detection of *BRAF* or *KRAS* mutations which were mutually exclusive. Three subtypes were MMR proficient: those with mutations in *BRAF* (6.9% of samples), mutations in *KRAS* (35%), or tumors lacking either *BRAF* or *KRAS* mutations (49%). Two subtypes were MMR deficient: the sporadic type (6.8%) with *BRAF* mutation and/or or hypermethylation of *MLH1* and the familial type (2.6%), which lacked $BRAF^{V600E}$ or hypermethylation of *MLH1*. A higher percentage of MMR-proficient tumors with $BRAF^{V600E}$ were proximal (76%), high-grade (44%), N2 stage (59%), and detected in women (59%), compared with MMR-proficient tumors without $BRAF^{V600E}$ or KRAS mutations (33%, 19%, 41%, and 42%, respectively; all $P < .0001$). A significantly lower proportion of patients with MMR-proficient tumors with mutant *BRAF* (hazard ratio = 1.43; 95% confidence interval: 1.11–1.85; $P_{adjusted} = .0065$) or mutant *KRAS* (hazard ratio = 1.48; 95% confidence interval: 1.27–1.74; $P_{adjusted} < .0001$) survived disease-free for 5 years compared with patients whose MMR-proficient tumors lacked mutations in either gene. Disease-free survival rates of patients with MMR-deficient sporadic or familial subtypes was similar to those of patients with MMR-proficient tumors without *BRAF* or KRAS mutations. The observed differences in survival rates of patients with different tumor subtypes were validated in an independent cohort. **CONCLUSIONS:** We identified subtypes of stage III colon cancer, based on detection of mutations in *BRAF* (V600E) or *KRAS*, and MMR status that show differences in clinical and pathologic features and disease-free survival. Patients with MMR-proficient tumors and *BRAF* or KRAS mutations had statistically shorter survival times than patients whose tumors lacked these mutations. The tumor subtype found in nearly half of the study cohort (MMR-proficient without $BRAF^{V600E}$ or KRAS mutations) had similar outcomes to those of patients with MMR-deficient cancers.

Keywords: Colorectal Cancer; Oncogene; Prognostic Factor; Genetics.

Colorectal cancer (CRC) is a biologically heterogeneous disease that develops via distinct pathways involving combinations of genetic and epigenetic changes.[1] Defining tumor subtypes based on pathway-driven alterations[2] has the potential to improve prognostication and guide targeted therapy. Two well-described pathways of colorectal tumorigenesis include chromosomal instability (CIN) and microsatellite instability (MSI), the latter being a consequence of deficient DNA mismatch repair (dMMR).[1,3] Deficient MMR can result from a germline mutation in an MMR gene (MLH1, MSH2, MSH6, PMS2), ie, Lynch syndrome (LS). More commonly, dMMR is sporadic and is due to epigenetic inactivation of *MLH1* that is generally associated with hypermethylation of promoter regions of cancer-specific genes known as the CpG island methylator phenotype (CIMP) high.[3–5] Sporadic dMMR, but not LS, tumors frequently carry the activating somatic V600E mutation in exon 15 of the *BRAF* oncogene.[6] BRAF is a member of the Raf kinase family that is a regulator of the MAP kinase/ERK signaling pathway.[7,8] $BRAF^{V600E}$ mutations occur downstream from and are mutually exclusive of *KRAS* codon 12 and 13 mutations[8] that are detected in 30%–40% of CRCs.[9] Both sporadic and LS-associated cancers with dMMR display a clinical

Abbreviations used in this paper: **CI, confidence interval; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CRC, colorectal cancer; DFS, disease-free survival; dMMR, deficient DNA mismatch repair; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; 5-FU, 5-fluorouracil; HR, hazard ratio; LS, Lynch syndrome; MSI, microsatellite instability; pMMR, proficient DNA mismatch repair.**

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phenotype characterized by right-sided location, high-grade histology, and abundant tumor-infiltrating lymphocytes.[10,11]

The association of *BRAF*, *KRAS*, and MMR individually with prognosis has been studied in colon cancers by ourselves[11–13] and others.[4,9,14–19] However, development of a classifier using biomarker combinations has the potential to identify distinct tumor subtypes with varying prognoses. Knowledge of pathways of colorectal tumorigenesis supports the subtyping of colon cancers using data for dMMR/MSI, *MLH1* methylation or CIMP, and mutations in $BRAF^{V600E}$ and *KRAS* oncogenes as proposed previously.[2,20] Tumor classification with these biomarkers includes serrated pathway subtypes in addition to subtypes reflecting the more typical adenoma-to-carcinoma sequence.[2,21] CRCs developing via a traditional adenoma–carcinoma sequence are characterized by CIN, lack of dMMR/MSI, and carry normal copies of *BRAF* and *KRAS* genes.[1] An alternate pathway is described where *KRAS* mutations develop as an early event in proficient MMR cancers.[2,20] Sporadic CRCs can also develop via a serrated neoplasia pathway, named for the pattern of crypts in precursor polyps, that is characterized by $BRAF^{V600E}$ mutations and CIMP-high. Cancers arising via this pathway can have deficient or proficient MMR, depending on the methylation status of the *MLH1* gene.[21] In contrast to sporadic dMMR cancers,[21] less is known about the prognosis of proficient DNA mismatch repair (pMMR) colon cancers that carry $BRAF^{V600E}$ mutations arising via a serrated pathway.[22] CRCs with dMMR that carry nonmutated copies of *BRAF* and lack *MLH1* methylation can be classified as "familial," as they are consistent with cancers arising in LS.[6] While molecular diversity among these pathways may result in differences in outcome, studies examining subtype classifications are limited to a report in all stages of CRC using the Surveillance, Epidemiology, and End Results Program registry from Washington state[23] and a modest-sized cohort of women.[20]

In patients undergoing surgical resection of CRC with curative intent, decision making for adjuvant chemotherapy is based entirely on clinical stage (TNM system), which provides an estimate of patient prognosis.[24] However, extensive intrastage variability in outcomes is observed that cannot be accurately predicted by the TNM staging system. Accordingly, more accurate prognostic classifiers are needed to further refine staging beyond TNM that can be readily implemented into clinical care. Such classifiers are ideally studied in a clinical trial cohort of same stage patients that meet strict eligibility requirements and receiving uniform treatment. Most published studies of molecular markers and prognosis evaluated 5-fluorouracil (5-FU)–based adjuvant therapy, and very limited data are available from patients treated with the current standard adjuvant regimen of 5-FU, leucovorin, and oxaliplatin (FOLFOX).[25] This is an important issue in that treatment-related interactions with biomarkers may exert modifying effects that can be reflected in patient survival rates.

In this report, prospectively collected stage III colon cancers from participants in a completed adjuvant chemotherapy trial of FOLFOX (NCCTG N0147; Alliance)[26] were classified into molecular subtypes using data for $BRAF^{V600E}$ and *KRAS* oncogenes, MMR protein expression, and *MLH1* methylation. We then characterized the prespecified subtypes with respect to clinicopathologic features and disease-free survival (DFS) rates.

Materials and Methods

Study Population

Patients with resected, stage III (any T, N1 or N2, M0) colonic adenocarcinomas participated in a phase III randomized trial of mFOLFOX6 or mFOLFOX6 + cetuximab (NCCTG N0147).[26] The current analysis includes all cancers with prospectively determined wild-type or mutated *KRAS*. Data for *KRAS*, *BRAF*, *MLH1* methylation, and MMR status were available on 2720 patients. A central pathology review was performed. Stratification factors included: number of metastatic regional lymph nodes (N1: 1–3 vs N2: ≥4), histologic grade (high: poorly differentiated/undifferentiated] vs low: well/moderately differentiated), and T stage. Proximal tumor site included cecum, ascending, hepatic flexure, and transverse colon; distal site included splenic flexure, descending and sigmoid colon. The study was approved by the Mayo Clinic Institutional Review Board and the North Central Cancer Treatment Group (NCCTG; now part of Alliance for Clinical Trials in Oncology). Each participant signed an Institutional Review Board–approved informed consent in accordance with current guidelines. Data quality was ensured by review by the Alliance Statistics and Data Center. All authors had access to the study data and reviewed and approved the final manuscript.

BRAF *and* KRAS *Gene Mutations*

Mutation status was determined using genomic DNA extracted from macrodissected, formalin-fixed, paraffin-embedded tumor tissue that contained at least 60% tumor cells. Testing for the c.1799T>A p.V600E *BRAF* mutation in exon 15 was performed using a multiplex allele-specific, real-time polymerase chain reaction–based assay and an automated sequencing technique.[27] Primer sequences included: wild-type forward [NED-TGATTTTGGTCATGCTACAGT]; mutant forward [6-Fam-CAGTGATTTTGCTCTAGCTTCAGA]; and reverse [GTTTCTTTCTAGTAACTCAGCAGC]. *KRAS* mutation status in exon 2 was analyzed in extracted DNA using the DxS mutation test kit KR-03/04 (DxS, Manchester, UK), assessing for 7 different mutations in codons 12 and 13.[28] For both genes, mutational analysis was performed in a Clinical Laboratory Improvement Amendments–compliant laboratory at Mayo Clinic.

DNA Mismatch Repair Proteins

MMR protein (MLH1, MSH2, and MSH6) expression was analyzed in formalin-fixed, paraffin-embedded tumor sections as described previously.[12] MMR protein loss was defined as absence of nuclear staining in tumor cells but positive nuclear staining in normal colonic epithelial cells and lymphocytes. Expression was scored by a gastrointestinal pathologist (TCS). Tumors were categorized as having dMMR if loss of at least one MMR protein was detected and pMMR if all proteins were intact.

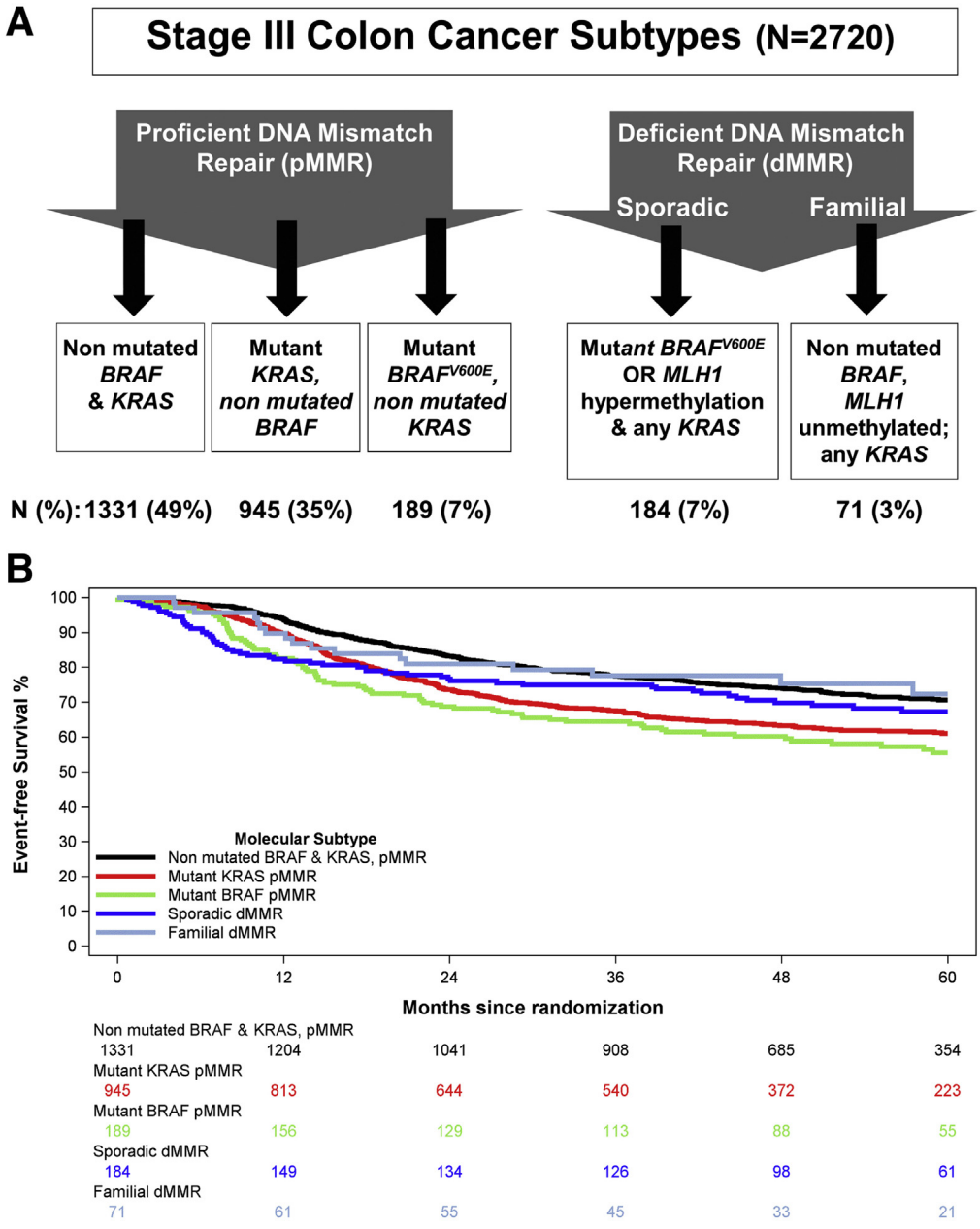

Figure 1. (*A*) Stage III colon cancers were categorized into 5 subtypes based on mutations in *BRAF (V600E)* and *KRAS (exon 2)* and MMR status. *BRAF*^{V600E} and *KRAS* mutations were mutually exclusive. (*B*) DFS is shown by molecular subtype in patients treated in an adjuvant trial of FOLFOX-based chemotherapy.

Analysis of MLH1 *Methylation*

Promoter methylation of *MLH1* was determined in *BRAF* nonmutated tumors in an effort to distinguish sporadic from familial dMMR patients. Tumor DNA was extracted from formalin-fixed, paraffin-embedded tissue and bisulfite modified using the EZ DNA Methylation Kit (Zymo Research Corp., Irvine, CA). Polymerase chain reaction primers were designed to detect differences between methylated and unmethylated DNA for the *hMLH1* promoter, as described.[29] Primers for the methylation-specific polymerase chain reaction assay included: methylated reaction (5′-FAM-AACGAATTAATAGGAAGAGCGGA TAGCG-3′; 5′-CGTCCCTCCCTAAAACGACTACTACCC-3′), unmethylated reaction (5′-NED-taaaaatgaattaataggaagagtggatagtg-3′; 5′-AATCTCTTCATCCCTCCCTAAAACA-3′); polymerase chain reaction products from these reactions were pooled (1:1 ratio) and diluted 1:13 using the GeneScan 400HD ROX Size Standard (Life Technologies, Foster City, CA) and formamide. Samples were run on an ABI 3100 Analyzer and data were analyzed using Genotyper software (Applied Biosystems, Foster City, CA).

Molecular Subtype Classifications

Tumors were categorized into 5 subtypes based on pathway-based classifications[2,20,21] using MMR status and mutations in *BRAF*^{V600E} or *KRAS*, which were mutually exclusive (Figure 1). We identified 3 pMMR subtypes: mutant *BRAF*^{V600E}, mutant *KRAS*, or tumors lacking a mutation in either *BRAF*^{V600E} or *KRAS*. Two subtypes were dMMR: sporadics with mutant *BRAF*^{V600E} or hypermethylation of *MLH1*, or familial, which lack *BRAF* mutations or hypermethylation of *MLH1*, and have any *KRAS* status.

Table 1. Demographic and Clinicopathologic Features by Molecular Subtype

	pMMR			dMMR			
	Nonmutated *BRAF/KRAS* (n = 1331)	Mutant *KRAS* (n = 945)	Mutant *BRAF*^{V600E} (n = 189)	Sporadic (n = 184)	Familial (n = 71)	Total (n = 2720)	*P* value[a]
Age, *y*							<.0001[b]
Median	57.00	59.00	63.00	66.00	46.00	58.00	
Range	19.00–84.00	22.00–85.00	31.00–81.00	36.00–86.00	28.00–74.00	19.00–86.00	
	Ref	.0006[c]	<.0001[c]	<.0001[c]	<.0001[c]		
		Ref	<.0001[c]	<.0001[c]	<.0001[c]		
			Ref	.0002[c]	<.0001[c]		
				Ref	<.0001[c]		
Age, n (%)							<.0001[d]
<50	343 (25.8)	205 (21.7)	15 (7.9)	8 (4.3)	45 (63.4)	616 (22.6)	
≥50	988 (74.2)	740 (78.3)	174 (92.1)	176 (95.7)	26 (36.6)	2104 (77.4)	
	Ref	.0250[d]	<.0001[d]	<.0001[d]	<.0001[d]		
		Ref	<.0001[d]	<.0001[d]	<.0001[d]		
			Ref	.1497[d]	<.0001[d]		
				Ref	<.0001[d]		
Sex, n (%)							<.0001[d]
Female	558 (41.9)	459 (48.6)	111 (58.7)	127 (69.0)	27 (38.0)	1282 (47.1)	
Male	773 (58.1)	486 (51.4)	78 (41.3)	57 (31.0)	44 (62.0)	1438 (52.9)	
	Ref	.0017[d]	<.0001[d]	<.0001[d]	.5166[d]		
		Ref	.0108[d]	<.0001[d]	.0863[d]		
			Ref	.0387[d]	.0029[d]		
				Ref	<.0001[d]		
Race, n (%)							<.0001[d]
White	1121 (85.8)	790 (85.9)	181 (95.8)	166 (91.2)	65 (91.5)	2323 (87.0)	
Black or African American	86 (6.6)	89 (9.7)	3 (1.6)	12 (6.6)	2 (2.8)	192 (7.2)	
Asian	84 (6.4)	35 (3.8)	4 (2.1)	4 (2.2)	2 (2.8)	129 (4.8)	
Other	16 (1.2)	6 (0.7)	1 (0.5)	0 (0.0)	2 (2.8)	25 (0.9)	
	Ref	.0016[d]	.0021[d]	.0548[d]	.1897[d]		
		Ref	.0015[d]	.2244[d]	.0504[e]		
			Ref	.0452[e]	.3055[e]		
				Ref	.1069[e]		
T stage, n (%)							.0005[d]
T1 or T2	227 (17.1)	143 (15.1)	20 (10.6)	17 (9.2)	5 (7.0)	412 (15.2)	
T3	974 (73.2)	665 (70.4)	139 (73.5)	140 (76.1)	55 (77.5)	1973 (72.6)	
T4	130 (9.8)	136 (14.4)	30 (15.9)	27 (14.7)	11 (15.5)	334 (12.3)	
	Ref	.0025[d]	.0063[d]	.0065[d]	.0398[d]		
		Ref	.2558[d]	.1062[d]	.1748[d]		
			Ref	.8446[d]	.6759[d]		
				Ref	.8511[d]		
N stage, n (%)							<.0001[d]
1–3	782 (58.8)	578 (61.2)	77 (40.7)	112 (60.9)	42 (59.2)	1591 (58.5)	
≥4	549 (41.2)	367 (38.8)	112 (59.3)	72 (39.1)	29 (40.8)	1129 (41.5)	
	Ref	.2477[d]	<.0001[d]	.5842[d]	.9465[d]		
		Ref	<.0001[d]	.9402[d]	.7378[d]		
			Ref	.0001[d]	.0079[d]		
				Ref	.8019[d]		
Grade, n (%)							<.0001[d]
High	257 (19.3)	186 (19.7)	84 (44.4)	100 (54.3)	36 (50.7)	663 (24.4)	
Low	1074 (80.7)	759 (80.3)	105 (55.6)	84 (45.7)	35 (49.3)	2057 (75.6)	
	Ref	.8244[d]	<.0001[d]	<.0001[d]	<.0001[d]		
		Ref	<.0001[d]	<.0001[d]	<.0001[d]		
			Ref	.0558[d]	.3670[d]		
					.6011[d]		

Table 1. Continued

	pMMR			dMMR			
	Nonmutated *BRAF/KRAS* (n = 1331)	Mutant *KRAS* (n = 945)	Mutant $BRAF^{V600E}$ (n = 189)	Sporadic (n = 184)	Familial (n = 71)	Total (n = 2720)	*P* value[a]
Tumor location, n (%)							<.0001[d]
Proximal	437 (33.2)	540 (58.1)	140 (75.7)	174 (95.1)	57 (86.4)	1348 (50.3)	
Distal	880 (66.8)	389 (41.9)	45 (24.3)	9 (4.9)	9 (13.6)	1332 (49.7)	
	Ref	<.0001[d]	<.0001[d]	<.0001[d]	<.0001[d]		
		Ref	<.0001[d]	<.0001[d]	<.0001[d]		
			Ref	<.0001[d]	.0697[d]		
				Ref	.0171[e]		
Tumor subsite location, n (%)							<.0001[d]
Cecum	152 (12.0)	259 (28.9)	46 (26.1)	55 (31.8)	19 (31.1)	531 (20.7)	
Ascending colon	138 (10.9)	154 (17.2)	44 (25.0)	56 (32.4)	14 (23.0)	406 (15.8)	
Hepatic flexure	36 (2.9)	27 (3.0)	15 (8.5)	28 (16.2)	10 (16.4)	116 (4.5)	
Transverse colon	72 (5.7)	74 (8.3)	27 (15.3)	26 (15.0)	9 (14.8)	208 (8.1)	
Splenic flexure	50 (4.0)	41 (4.6)	6 (3.4)	1 (0.6)	2 (3.3)	100 (3.9)	
Descending colon	79 (6.3)	49 (5.5)	8 (4.5)	1 (0.6)	0 (0.0)	137 (5.3)	
Sigmoid colon	735 (58.2)	291 (32.5)	30 (17.0)	6 (3.5)	7 (11.5)	1069 (41.6)	
	Ref	<.0001[d]	<.0001[d]	<.0001[d]	<.0001[d]		
		Ref	<.0001[d]	<.0001[d]	<.0001[d]		
			Ref	<.0001[e]	.3361[d]		
				Ref	.1415[d]		

[a]*P* values are unadjusted.
[b]*P* value based on Kruskal–Wallis test.
[c]*P* value based on Wilcoxon rank sum test.
[d]*P* value based on χ^2 test.
[e]*P* value based on Fisher's exact test.

Validation Cohort

To validate the prognostic utility of our subtype classifier, we examined an independent cohort of stage III colon carcinoma patients (N = 783) obtained from the Sage Bionetworks (Seattle, WA) consortium that consist of case series and a clinical trial cohort of well-annotated colon cancer patients with extended follow-up. Among these patients, 688 of 738 (93.2%) had received 5-FU–based adjuvant chemotherapy and of these 473 (64%) received 5-FU/leucovorin ± irinotecan in an adjuvant study (PETACC-3). Survival data were censored at 5 years with median follow-up of 6.1 years; 269 DFS events were observed. Data for *KRAS* and $BRAF^{V600E}$ mutations and MMR status, determined by MMR protein expression or MSI, were used to classify patient tumors into the molecular subtypes as evaluated here. Deficient MMR tumors were divided based on *BRAF* status alone because data for *MLH1* methylation were not available.

Statistical Methods

All biomarker data were analyzed with investigators blinded to patient outcomes. For patients who were alive and disease-free, DFS was censored at the earlier date of last disease evaluation or 5 years post randomization. Analysis of the primary study end point of DFS, defined as time from date of randomization to first documented disease recurrence or death (due to all causes), whichever occurred first, was reported previously.[26] The 2 study arms were pooled given the lack of statistically significant differences in DFS rates,[26] and the lack of a significant interaction ($P > .38$) between treatment and any of the biomarkers (ie, *KRAS, BRAF*, MMR) or the 5-level molecular subtype classification. Kruskal–Wallis (or Wilcoxon rank-sum) and χ^2 (or Fisher's exact) tests were used to compare continuous and categorical variables, respectively, among the 5 subtypes. Median follow-up for surviving patients was 4.9 years (range, 0.0–8.4 years). Kaplan-Meier methods were used to describe the distributions of DFS.[30] Univariate Cox proportional hazard models[31] were used to explore the associations of patient characteristics and biomarkers with DFS. Thereafter, multivariable Cox models were utilized and unless otherwise specified, all models were adjusted for stratification factors selected a priori[12] (provided here) and 95% confidence intervals (CIs) are provided for all hazard ratios. Two-sided *P* values are reported and, in general, values <.05 were considered statistically significant. An effort to control for multiple comparisons was made during the planning stage by using well-established biomarkers whose classification is supported by the literature.[2,20] Analyses were performed using SAS version 9.3 (SAS Institute Inc, Cary, NC) and R version 2.14.[32] Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center.

Results

Tumor Subtype and Clinical and Pathologic Characteristics

Among the 2720 cases with complete data on all tumor markers, tumors were classified into 3 pMMR subtypes

that included tumors with mutations in either $BRAF^{V600E}$ (n = 189; 6.9%) or *KRAS* (n = 945; 34.7%), and those lacking a mutation in these genes (n = 1331; 48.9%) (Table 1; Figure 1*A*). Of note, mutations in $BRAF^{V600E}$ and *KRAS* were mutually exclusive. The 2 dMMR subtypes included sporadic (n = 184; 6.8%) tumors with $BRAF^{V600E}$ mutations and/or *MLH1* hypermethylation, and familial (n = 71; 2.6%) cancers that lacked $BRAF^{V600E}$ mutations and had unmethylated *MLH1*, which is consistent with LS (Table 1; Figure 1*A*). Among pMMR subtypes, patients with $BRAF^{V600E}$ mutated tumors were oldest (median age, 63 years), were most likely to be women (58.7%), and had the highest rates of proximal site (75.7%), T4 stage (15.9%), high-grade histology (44.4%), and N2 stage (59.3%) (Table 1). MMR-proficient tumors of the mutant *KRAS* subtype were more commonly located in the proximal colon (58.1% vs 33.2%) compared with tumors lacking mutations in $BRAF^{V600E}$ or *KRAS* (Table 1). Within the most prevalent subtype of pMMR tumors lacking mutations in either $BRAF^{V600E}$ or *KRAS*, there were more men than women compared with the other subtypes ($P \leq .002$), except for familial dMMR patients, and 66.8% of tumors were located in the distal colon (Table 1). Patients with sporadic dMMR tumors had the oldest median age (66 years) at randomization among all subytpes, were most likely from women (69.0%), had highest rate of high-grade histology (54.3%), and nearly all (95.1%) were located in the proximal colon (Table 1). The familial subtype of dMMR tumors was associated with younger age, male sex, high-grade histology, and proximal site, which are features of LS-associated colon cancers[33] (Table 1). Among colon cancers with loss of MLH1 protein expression, 80% had $BRAF^{V600E}$ mutations and the remaining cases had nonmutated *BRAF* with promoter hypermethylation of *MLH1*.

The distributions of the 5 subtypes in relation to tumor subsite location (ie, cecum, ascending colon hepatic flexure, transverse colon, splenic flexure, descending colon, and sigmoid colon) were examined (Table 1). A majority of pMMR tumors with $BRAF^{V600E}$ mutations were located in the proximal colon (75.7%), with approximately half (51.1%) found in the cecum plus ascending colon. Nearly half (46.1%) of cancers of the mutated *KRAS* subtype were located in the cecum plus ascending colon; one third (32.5%) were located in the sigmoid colon. Proficient MMR tumors lacking $BRAF^{V600E}$ or *KRAS* mutations were frequently located in the sigmoid colon (58.2%), which is typical of the CIN pathway.[1] Sporadic or familial dMMR subtypes showed a predilection for the proximal colon that also included higher rates of hepatic flexure and transverse colon location compared with pMMR cancers.

Tumor subtype was examined in relationship to patient race (ie, white, African American, or Asian). Compared to the other tumor subtypes, African Americans had the highest representation among the mutated *KRAS* and pMMR subtype (Table 1). Asian patients were most likely to have pMMR tumors lacking mutations in $BRAF^{V600E}$ or *KRAS* and in contrast to African Americans or whites, were more frequently represented among familial vs sporadic dMMR tumors.

Tumor Subtype and Disease-Free Survival

Distributions of DFS rates are shown in Kaplan-Meier curves across the 5 tumor subtypes (Figure 1*B*) and 5-year DFS rates are provided (Table 2). The 5-year DFS rates for the 3 pMMR subtypes range from 55.5% (95% CI: 48.0%−62.9%) for $BRAF^{V600E}$ mutant, 61% (95% CI: 57.6%−64.4%) for *KRAS* mutant, and 70.7% (95% CI: 68.0%−73.3%) for tumors lacking mutations in either gene (Table 2). DFS was not statistically different for pMMR tumors with mutations in $BRAF^{V600E}$ or in *KRAS* ($P_{\text{unadjusted}} = .1486$). Compared with the poorer outcome of

Table 2. Five-Year Disease-Free Survival Rates by Molecular Subtype

	pMMR			dMMR		
	Nonmutant *BRAF/KRAS*	Mutant *KRAS*	Mutant $BRAF^{V600E}$	Sporadic	Familial	*P* value[a]
All patients						<.0001
Events/n	347/1331	333/945	80/189	56/184	17/71	
5-Year rate, % (95% CI)	70.7 (68.0–73.3)	61.0 (57.6–64.4)	55.5 (48.0–62.9)	67.3 (60.1–74.5)	72.3 (60.6–84.1)	
Proximal tumor						.0005
Events/n	138/437	198/540	67/140	53/174	11/57	
5-Year rate, % (95% CI)	65.0 (60.1–69.8)	59.2 (54.7–63.7)	50.9 (42.4–59.4)	67.3 (59.9–74.7)	77.6 (65.2–90.0)	
Distal tumor						.0012
Events/n	204/880	132/389	13/45	3/9	4/9	
5-Year rate, % (95% CI)	73.7 (70.5–76.9)	62.8 (57.6–68.0)	66.0 (49.9–82.0)	66.7 (35.9–97.5)	38.9 (0.0–79.3)	
N1 tumor						<.0001
Events/n	143/782	162/578	23/77	21/112	7/42	
5-Year rate, % (95% CI)	79.4 (76.3–82.5)	67.9 (63.7–72.2)	68.2 (57.2–79.1)	79.3 (71.3–87.4)	82.4 (70.4–94.3)	
N2 tumor						.0012
Events/n	204/549	171/367	57/112	35/72	10/29	
5-Year rate, % (95% CI)	58.2 (53.7–62.8)	50.1 (44.7–55.5)	46.5 (36.6–56.5)	48.4 (36.3–60.5)	57.5 (35.9–79.1)	

[a]Unadjusted Cox model.

the $BRAF^{V600E}$ and *KRAS* mutant subtypes, favorable DFS was observed for pMMR tumors lacking mutations in either gene (vs mutant $BRAF^{V600E}$: hazard ratio [HR] = 0.56; 95% CI: 0.44–0.72; vs mutant *KRAS*: HR = 0.67; 95% CI: 0.58–0.78; $P_{\text{unadjusted}} < .0001$ for both). In addition, DFS for the pMMR subtype without $BRAF^{V600E}$ or *KRAS* mutations did not differ significantly from the sporadic ($P_{\text{unadjusted}} = .1448$) or familial ($P_{\text{unadjusted}} = .8511$) dMMR subtypes (Table 3). Five-year DFS rates for sporadic and familial dMMR subtypes were 67.3% (95% CI: 60.1%−74.5%) and 72.3% (95% CI: 60.6%−84.1%), respectively, and were not statistically different (Tables 2 and 3). Overall, the univariate results were maintained in a multivariable analysis after adjustment for multiple covariates (Table 3).

An earlier study in this clinical trial cohort found that tumor site and N stage significantly altered the relationship between MMR status and DFS.[12] Accordingly, we evaluated the prognostic impact of the 5 subtypes stratified by tumor site and N stage. Although the interaction tests did not achieve statistical significance likely due to limited power (tumor site: $P_{\text{adjusted}} = .1368$; N stage: $P_{\text{adjusted}} = .1103$), we found that results in the overall cohort were maintained in proximal cancers indicated by lack of significant differences in DFS. Among proximal tumors, 5-year DFS rates for patients with pMMR tumors lacking mutations in $BRAF^{V600E}$ and *KRAS* or for both dMMR subtypes were significantly better than rates for $BRAF^{V600E}$ mutated or *KRAS* mutated pMMR subtypes (Tables 2 and 4). Patients with proximal dMMR tumors of the familial subtype had the highest 5-year DFS rate (77.6%; Table 2) that did not differ significantly from dMMR tumors of the sporadic subtype or pMMR tumors lacking $BRAF^{V600E}$ and *KRAS* mutations (Table 4). Of note, DFS for dMMR tumors of the familial subtype was poorer among distal vs proximal tumors (Table 2; Figure 2*A* and *B*). Among distal pMMR cancers, statistically significant differences in DFS were found only for *KRAS*-mutated tumors (vs those without *KRAS* and *BRAF* mutations), yet statistical power was limited (Table 4). A trend toward better DFS was found in distal vs proximal tumors with $BRAF^{V600E}$ mutations and tumors without $BRAF^{V600E}$ or *KRAS* mutations (Table 2).

Among patients with N1 tumors, the association of tumor subtypes with DFS did not differ significantly from the overall cohort (Table 2 and Figures 1*B* and 2*C*). Among patients with N2 tumors, however, poor DFS was observed for dMMR tumors of the sporadic subtype (Table 2, Figure 2*D*) that did not differ significantly from DFS of pMMR subtypes with mutated *KRAS* ($P_{\text{adjusted}} = .9195$) or mutated $BRAF^{V600E}$ ($P_{\text{adjusted}} = .8231$) (Table 4). In contrast, N1 tumors of the dMMR sporadic subtype had DFS rates that were significantly improved compared with DFS of patients with pMMR mutated *KRAS* tumors (HR = 0.51; 95% CI: 0.31–0.82; $P_{\text{adjusted}} = .0054$), or showed a strong trend vs the mutated $BRAF^{V600E}$ (HR = 0.50; 95% CI: 0.28–0.91; $P_{\text{adjusted}} = .0238$) subtype (Table 4 and Figure 2*C*).

External Validation Cohort

We attempted to validate the prognostic utility of our classifier in an independent cohort of stage III colon cancer patients treated with 5-FU–based adjuvant chemotherapy. Patients from this external cohort were categorized into the same molecular subtypes as in our dataset, with the exception that dMMR tumors were divided based on *BRAF* status alone (see Materials and Methods). In this independent cohort, a statistically significant difference was seen among the 5 molecular subtypes ($P = .014$) as was demonstrated in the primary N0147 cohort (Figure 3). A similarly favorable outcome for pMMR tumors lacking $BRAF^{V600E}$ or *KRAS* mutations and dMMR tumors was observed. In addition, poorer DFS among patients with $BRAF^{V600E}$ mutant or *KRAS* mutant pMMR cancers was observed as reflected in their 5-year DFS rates (Figure 3, Table 2). Accordingly, the key prognostic findings of our biomarker classifier were validated.

Discussion

In patients undergoing surgical resection of CRC, prognosis and management are based entirely on the TNM

Table 3. Associations Between Molecular Subtypes and Disease-Free Survival

Pair-wise comparisons	Univariate		Multivariate[a]	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
pMMR: Mut *KRAS* vs nonmutated *KRAS/BRAF*	1.488 (1.281–1.730)	<.0001	1.483 (1.267–1.736)	<.0001
pMMR: Mut $BRAF^{V600E}$ vs nonmutated *KRAS/BRAF*	1.782 (1.397–2.272)	<.0001	1.430 (1.105–1.849)	.0065
pMMR: Sporadic dMMR vs nonmutated *KRAS/BRAF*	1.234 (0.930–1.636)	.1448	1.090 (0.801–1.482)	.5850
Familial dMMR vs pMMR, nonmutated *KRAS/BRAF*	0.954 (0.587–1.553)	.8511	0.770 (0.452–1.309)	.3339
pMMR: Mut $BRAF^{V600E}$ vs Mut *KRAS*	1.197 (0.938–1.528)	.1486	0.964 (0.748–1.242)	.7763
Sporadic dMMR vs pMMR, Mut *KRAS*	0.829 (0.624–1.100)	.1938	0.735 (0.545–0.991)	.0432
Familial dMMR vs pMMR, Mut *KRAS*	0.641 (0.394–1.044)	.0739	0.519 (0.306–0.881)	.0151
Sporadic dMMR vs pMMR, Mut $BRAF^{V600E}$	0.692 (0.492–0.974)	.0349	0.762 (0.539–1.077)	.1240
Familial dMMR vs pMMR, mut $BRAF^{V600E}$	0.536 (0.317–0.904)	.0194	0.538 (0.306–0.946)	.0312
Familial dMMR vs sporadic dMMR	0.774 (0.450–1.331)	.3541	0.706 (0.394–1.267)	.2435

Mut, mutant.
[a]Adjusted for age, sex, T and N stage, grade, number of lymph nodes examined, tumor location, treatment.

Table 4. Multivariable Associations[a] Between Molecular Subtypes and Disease-Free Survival Stratified by Tumor Location and N Stage

Pair-wise comparisons	Tumor location				N stage			
	Proximal		Distal		N1		N2	
	HR (95% CI)	*P* value[b]	HR (95% CI)	*P* value	HR (95% CI)	*P* value[b]	HR (95% CI)	*P* value
pMMR: Mut *KRAS* vs nonmutated KRAS/*BRAF*	1.313 (1.053–1.637)	0.0155	1.658 (1.326–2.074)	<.0001	1.649 (1.301–2.090)	<.0001	1.367 (1.105–1.690)	.0039
pMMR: Mut $BRAF^{V600E}$ vs nonmutated *KRAS/BRAF*	1.432 (1.056–1.942)	0.0209	1.104 (0.626–1.948)	0.7318	1.665 (1.055–2.626)	0.0284	1.328 (0.970–1.819)	.0768
Sporadic dMMR vs pMMR, nonmutated *KRAS/BRAF*	1.004 (0.717–1.406)	0.9826	1.813 (0.574–5.728)	0.3107	0.836 (0.510–1.371)	0.4780	1.394 (0.938–2.072)	.1000
Familial dMMR vs pMMR, nonmutated KRAS/BRAF	0.602 (0.321–1.126)	0.1121	2.218 (0.810–6.072)	0.2380	0.804 (0.348–1.858)	0.6095	0.723 (0.363–1.439)	.3557
pMMR: Mut $BRAF^{V600E}$ vs Mut *KRAS*	1.091 (0.817–1.456)	0.5567	0.666 (0.373–1.188)	0.1688	1.010 (0.648–1.573)	0.9655	0.972 (0.711–1.329)	.8576
Sporadic dMMR vs pMMR, Mut *KRAS*	0.764 (0.555–1.053)	0.1003	1.093 (0.344–3.472)	0.8796	0.507 (0.314–0.818)	0.0054	1.020 (0.692–1.503)	.9195
Familial dMMR vs pMMR, Mut *KRAS*	0.458 (0.246–0.853)	0.0139	1.338 (0.484–3.695)	0.5747	0.488 (0.212–1.120)	0.0904	0.529 (0.266–1.053)	.0697
Sporadic dMMR vs pMMR, Mut $BRAF^{V600E}$	0.701 (0.486–1.010)	0.0566	1.642 (0.464–5.812)	0.4421	0.502 (0.276–0.913)	0.0238	1.050 (0.685–1.608)	.8231
Familial dMMR vs pMMR, Mut $BRAF^{V600E}$	0.420 (0.218–0.811)	0.0098	2.009 (0.642–6.282)	0.2306	0.483 (0.193–1.206)	0.1192	0.544 (0.265–1.117)	.0974
Familial dMMR vs sporadic dMMR	0.599 (0.306–1.173)	0.1352	1.223 (0.266–5.625)	0.7956	0.962 (0.379–2.440)	0.9344	0.518 (0.243–1.104)	.0885

Mut, mutant.
[a]Adjusted for age, sex, T and N stage, grade, number of lymph nodes examined, tumor location, treatment.
[b]Interaction between molecular subtypes and tumor location (adjusted $P_{interaction} = .1368$) or with N stage (adjusted $P_{interaction} = .1103$).

staging system,[24] despite considerable stage-independent variability in outcomes. Accordingly, prognostic classifiers that can be readily implemented into clinical practice are needed to enhance clinical decision making. In stage III colon cancers from a recent adjuvant chemotherapy trial,[26] we classified tumors into 5 prespecified subtypes using a biomarker combination of $BRAF^{V600E}$ and *KRAS* mutations, *MLH1* methylation, and MMR status. On the basis of this classification adapted from Jass,[2] we found statistically significant differences in clinicopathologic features and patient survival rates. Importantly, no interactions were found between biomarkers and treatment arms or between subtypes and DFS by treatment arm that permitted pooling of data for the study arms. Proficient MMR tumors that were nonmutated for $BRAF^{V600E}$ and *KRAS* were the most prevalent subtype and represented 49% of our study cohort. Two thirds of these tumors were located in the distal colon. This patient subtype had DFS rates that were significantly better than the other pMMR subtypes with mutated $BRAF^{V600E}$ or *KRAS*, which both showed relatively poor survival rates. In addition, the prognosis of pMMR tumors that were nonmutated for $BRAF^{V600E}$ and *KRAS* did not differ significantly from dMMR tumors of the sporadic or familial subtypes. When these tumors and the dMMR subtypes are considered together, 58% of our study patients had favorable survival.

We identified phenotypic features of the poorly characterized, pMMR subtype with $BRAF^{V600E}$ mutations whose frequency was found to be similar to the dMMR sporadic subtype. Compared with other pMMR subtypes, patients with mutant $BRAF^{V600E}$ tumors were older, more likely to be women, and had higher rates of high-grade histology and N2 stage. Patients with pMMR mutant, $BRAF^{V600E}$ tumors had a poor prognosis that did not differ significantly from that of the mutant *KRAS* subtype that lacked $BRAF^{V600E}$ mutations given their mutual exclusivity.[8] Importantly, the mutant $BRAF^{V600E}$ pathway leads to both pMMR and dMMR cancers,[21,34] with *MLH1* hypermethylation being the key event that confers dMMR which is associated with favorable prognosis.[35] Both mutant $BRAF^{V600E}$ pMMR and dMMR subtypes were strongly associated with proximal tumor site (76% and 95%, respectively). In contrast to CRCs with CIN that develop from typical colorectal adenomas.[1] $BRAF^{V600E}$ mutant and/or *MLH1* hypermethylated colon cancers are believed to develop from a precursor lesion known as the sessile serrated adenoma/polyp based on clinical and gene expression data.[21,36,37] Sessile serrated adenoma/polyps are found predominantly in the proximal colon, carry frequent $BRAF^{V600E}$ mutations, and are CIMP-high.[21] $BRAF^{V600E}$ is an early driver mutation that promotes tumor progression through methylation-induced *p16/Ink4a* inactivation.[38,39] Gene expression profiling of mutant $BRAF^{V600E}$ pMMR cancers reveals up-regulation of genes regulating epithelial mesenchymal transition and matrix remodeling that can facilitate tumor invasion and metastasis and, thereby, contribute to their poor outcome.[37]

Results in the overall cohort were maintained in proximal cancers as indicated by a lack of significant differences

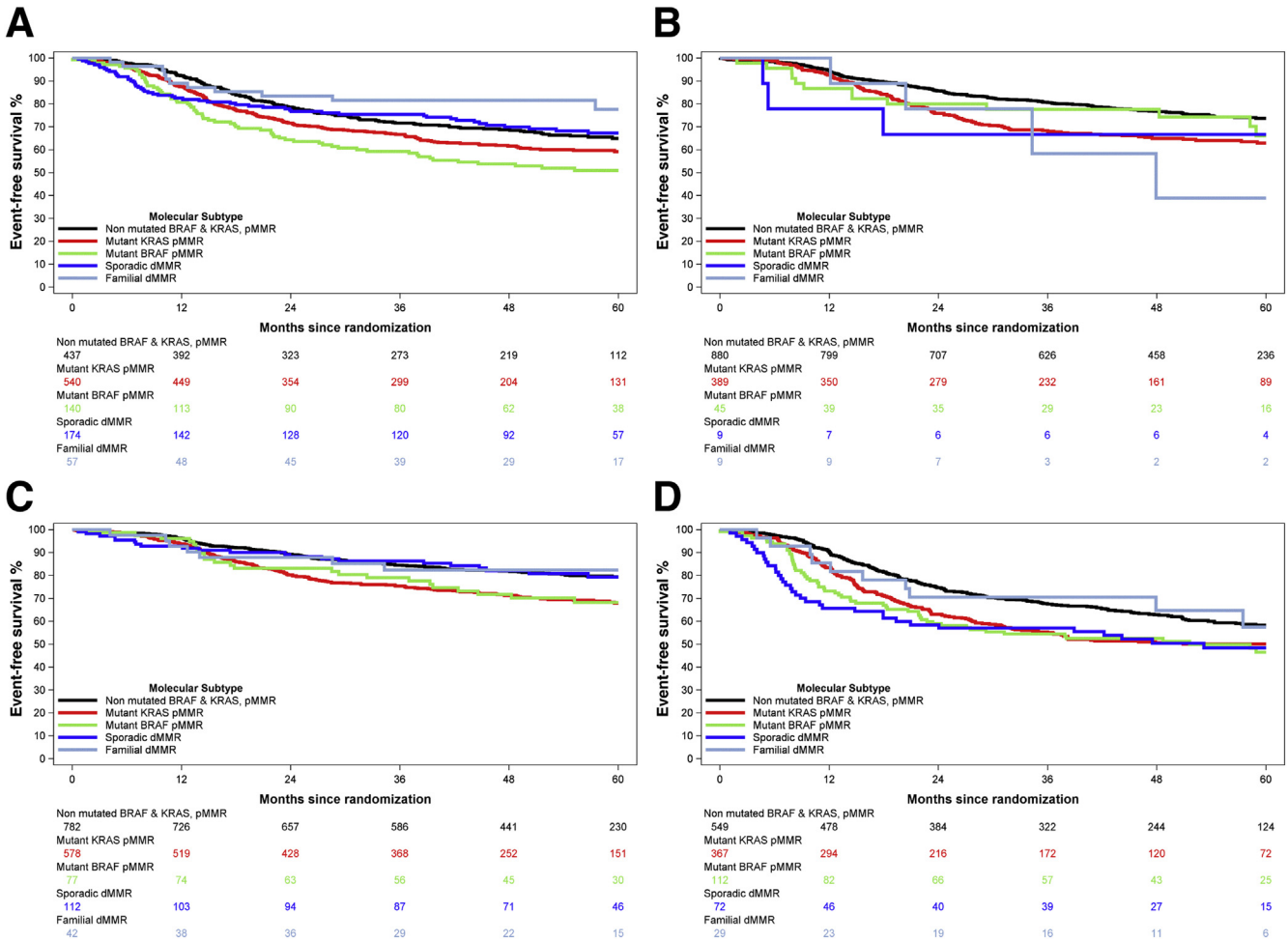

Figure 2. DFS by molecular subtypes among (*A*) proximal or (*B*) distal stage III colon cancers. DFS by molecular subtypes among patients with stage III colon cancer and (*C*) 1–3 metastatic regional lymph nodes (N1) or (*D*) 4 or more metastatic regional lymph nodes (N2).

in DFS. The observed DFS differences among distal tumors are of interest, yet statistical power was limited. We also examined the prognostic impact of our subtype classification by N stage. Within N1 cancers, we observed favorable 5-year DFS rates for pMMR tumors lacking $BRAF^{V600E}$ or *KRAS* mutations that did not differ significantly from both dMMR subtypes. These data raise the question of whether the survival of these 2 subtypes of stage III N1 patients treated with FOLFOX might be similar to a stage II population. In a review of data for stage II colon cancers from adjuvant chemotherapy trials that evaluated FOLFOX,[25,40–42] reported DFS rates are similar to those observed in our stage III N1 tumors without $BRAF^{V600E}$ or *KRAS* mutations or in the dMMR subtypes. This finding suggests that N1 pMMR tumors without $BRAF^{V600E}$ or *KRAS* mutations may have an intrinsically better prognosis irrespective of therapy, or alternatively, may receive greater benefit from FOLFOX vs the other subtypes. The situation in dMMR tumors is more complex given data suggesting lack of 5-FU benefit[43] and the unknown benefit, if any, of oxaliplatin combined with 5-FU/leucovorin in stage III dMMR patients.[19] Although the prognostic impact of molecular subtypes in N1 cancers was similar to the overall cohort, we unexpectedly observed poor DFS for N2 dMMR sporadic tumors, which was not significantly different from the poor prognosis of N2 pMMR tumors with mutant $BRAF^{V600E}$ or mutant *KRAS*. However, this finding was not observed among N2 dMMR tumors of the familial subtype that maintained their favorable HRs, and an explanation awaits further research.

The mutant *KRAS* pMMR subtype had the highest percentage of African Americans compared with the other subtypes, consistent with data indicating higher rates of *KRAS* mutations in CRCs from African Americans.[44,45] Conflicting data have been reported for the frequency of dMMR/MSI in CRCs from African Americans compared with whites,[45] yet our study does not demonstrate a difference in the rate of African Americans by MMR status. Our data for mutant *KRAS*, albeit preliminary due to small patient numbers of non-white race, suggest that colon cancers from African Americans may be associated with this poor prognostic subtype.

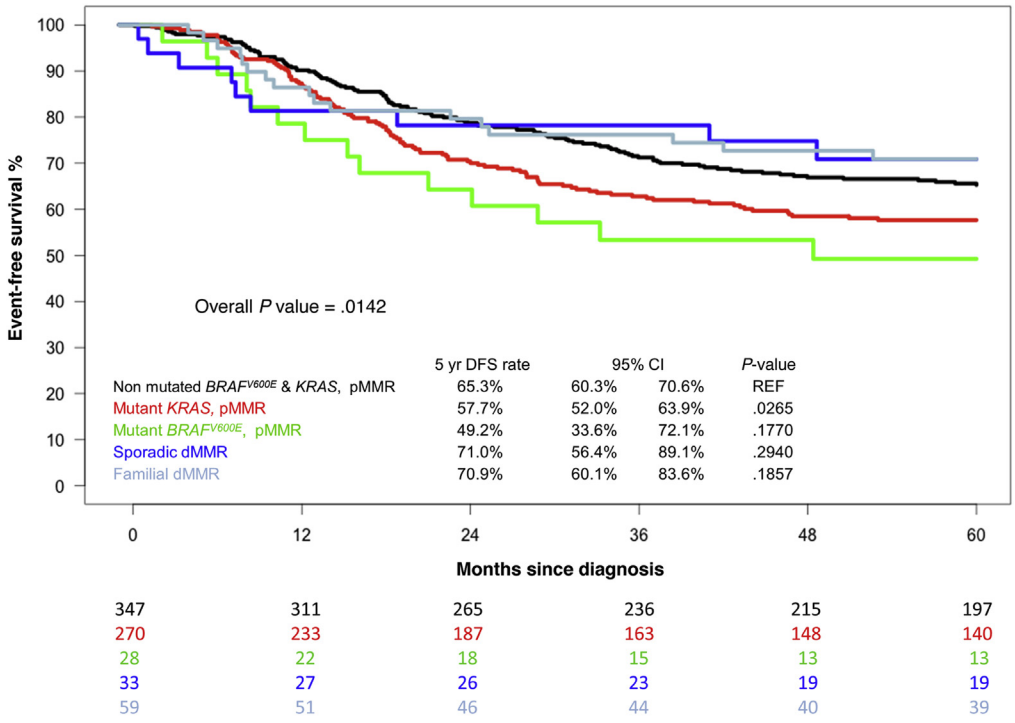

Figure 3. Independent cohort of stage III colon cancer patients used for external validation of the subtype classifier. DFS is shown for the individual subtypes.

Our findings support limited data demonstrating the ability of subtype classifications to predict clinical outcomes. Recently, a CRC subtype classification[20] was applied to tumor tissues from the Iowa Women's Health Study, which found differences in age at diagnosis, tumor site, and histologic grade across 3 CRC subtypes defined by combinations of MSI, CIMP, *BRAF*, and *KRAS* status. However, no statistically significant differences in survival were found across the tumor subtypes in this smaller cohort that was limited to women.[20] In contrast to our study, the authors defined a mutant $BRAF^{V600E}$ serrated subtype without regard to MSI status and did not distinguish the MSI-high familial subtype as a distinct group.[20] Data shown here and elsewhere[21,37] suggest that the serrated neoplasia pathway can give rise to colon cancer subtypes with divergent prognoses. Our subtype classification was more informative than analysis of individual biomarkers.[12] Among the biomarkers analyzed, dMMR status is the most extensively studied and has been associated with favorable survival in untreated patients and in those receiving 5-FU–based adjuvant therapy.[11,12,14,17,35] The prognostic impact of oncogenic *KRAS* in stage II and III colon cancers has been inconsistent,[9,12,14,17,46–48] and $BRAF^{V600E}$ mutations have generally been associated with adverse outcomes, particularly in metastatic CRCs.[12,14,15,18,47,49,50] Importantly, we were able to validate the key findings for the prognostic impact of our subtype classifier in an independent cohort of stage III colon cancer patients treated with 5-FU–based adjuvant chemotherapy. This finding supports the robustness of our classifier to detect clinically significant prognostic differences.

Patients in our study cohort were treated with the current standard adjuvant FOLFOX regimen, and only limited data are available for the prognostic impact of the biomarkers studied here in FOLFOX-treated patients.[12,19] Important strengths of our study include the large size of our clinical trial cohort with uniform treatment, meticulous follow-up data, and an external validation cohort. Our subtype classifier capitalizes on common testing for *KRAS* and *BRAF* status in clinical practice and the recommendation for universal MMR/MSI testing by the National Comprehensive Cancer Network. Limitations include the retrospective design and inability to examine the predictive potential of our subtype classifier with respect to treatment response. Although an effort was made to control for multiple comparisons during the study planning stage by utilizing well-established biomarkers whose classification was supported by the literature, pairwise comparisons with *P* values that are close to the .05 significance level should be interpreted with caution and their clinical significance considered. We acknowledge that other molecular events within the subtypes may indeed impact prognosis or chemosensitivity, which can contribute to the observed subtype-specific survival differences. A potential confounder is the use of aspirin or other nonsteroidal anti-inflammatory drugs and using questionnaire data that were available from a subset of the study population (n = 1757), no evidence was found to indicate that use of these drugs modified the association between subtypes and DFS.

In conclusion, we found that a biomarker-based classifier can identify prognostically distinct subtypes within stage III colon cancer patients that was externally validated. We identified a phenotype associated with $BRAF^{V600E}$ mutations and pMMR that was clinically aggressive as was the mutant *KRAS* subtype. The pMMR subtype without *BRAF* or *KRAS* mutations accounted for nearly half of our study

cohort and had a favorable prognosis that did not differ significantly from dMMR cancers. The finding that pMMR and dMMR subtypes with $BRAF^{V600E}$ mutations share certain clinical and pathologic characteristics, but have divergent prognoses underscores the importance of testing for MMR/MSI to distinguish them. The poor prognosis, pMMR subtype with mutated $BRAF^{V600E}$ can potentially be targeted if *BRAF* inhibitors can be rendered efficacious in CRCs by blocking rebound epidermal growth factor receptor activation.[51,52] Taken together, our biomarker classifier provides important prognostic information in stage III colon cancers with implications for patient management.

References

1. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology 2008;135:1079–1099.
2. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:113–130.
3. Sinicrope FA, Sargent DJ. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin Cancer Res 2012;18:1506–1512.
4. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 2009; 58:90–96.
5. Hinoue T, Weisenberger DJ, Pan F, et al. Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. PLoS One 2009; 4:e8357.
6. Domingo E, Niessen RC, Oliveira C, et al. BRAF-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional MLH1 and MSH2 genes. Oncogene 2005;24:3995–3998.
7. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–954.
8. Rajagopalan H, Bardelli A, Lengauer C, et al. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002;418:934.
9. Ogino S, Meyerhardt JA, Irahara N, et al. KRAS mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. Clin Cancer Res 2009; 15:7322–7329.
10. Jass JR, Do KA, Simms LA, et al. Morphology of sporadic colorectal cancer with DNA replication errors. Gut 1998;42:673–679.
11. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003;349:247–257.
12. Sinicrope FA, Mahoney MR, Smyrk TC, et al. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. J Clin Oncol 2013;31:3664–3672.
13. Sinicrope FA, Foster NR, Thibodeau SN, et al. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. J Natl Cancer Inst 2011;103:863–875.
14. Roth AD, Tejpar S, Delorenzi M, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 2010; 28:466–474.
15. Phipps AI, Buchanan DD, Makar KW, et al. BRAF mutation status and survival after colorectal cancer diagnosis according to patient and tumor characteristics. Cancer Epidemiol Biomarkers Prev 2012;21:1792–1798.
16. Phipps AI, Buchanan DD, Makar KW, et al. KRAS-mutation status in relation to colorectal cancer survival: the joint impact of correlated tumour markers. Br J Cancer 2013;108:1757–1764.
17. Samowitz WS, Curtin K, Schaffer D, et al. Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. Cancer Epidemiol Biomarkers Prev 2000;9:1193–1197.
18. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005; 65:6063–6069.
19. Gavin PG, Colangelo LH, Fumagalli D, et al. Mutation profiling and microsatellite instability in stage II and III colon cancer: an assessment of their prognostic and oxaliplatin predictive value. Clin Cancer Res 2012; 18:6531–6541.
20. Samadder NJ, Vierkant RA, Tillmans LS, et al. Associations between colorectal cancer molecular markers and pathways with clinicopathologic features in older women. Gastroenterology 2013;145:348–356. e1–2.
21. Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 2010; 138:2088–2100.
22. Bond CE, Umapathy A, Buttenshaw RL, et al. Chromosomal instability in BRAF mutant, microsatellite stable colorectal cancers. PLoS One 2012;7:e47483.
23. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77–87.
24. Greene FL, Page DL, Fleming ID, et al. AJCC cancer staging manual. 6th ed. Philadelphia, PA: Lippincott Raven Publishers, 2002:200.
25. Andre T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343–2351.
26. Alberts SR, Sargent DJ, Nair S, et al. Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. JAMA 2012; 307:1383–1393.
27. Domingo E, Laiho P, Ollikainen M, et al. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. J Med Genet 2004;41:664–668.
28. Angulo B, Garcia-Garcia E, Martinez R, et al. A commercial real-time PCR kit provides greater sensitivity than direct sequencing to detect KRAS mutations: a morphology-based approach in colorectal carcinoma. J Mol Diagn 2010;12:292–299.

29. Grady WM, Rajput A, Lutterbaugh JD, et al. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Cancer Res 2001;61:900–902.
30. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assn 1958;53:457–481.
31. Cox DR. Regression models and life-tables. J R Stat Soc 1972;34:187–220.
32. R Development Core Team: A Language and Environment for Statistical Computing. Vienna, Austria, R Foundation for Statistical Computing, 2011. Available at: http://www.R-project.org/. Accessed September, 2014.
33. Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology 2010;138:2073–2087. e3.
34. Kim YH, Kakar S, Cun L, et al. Distinct CpG island methylation profiles and BRAF mutation status in serrated and adenomatous colorectal polyps. Intl J Cancer 2008;123:2587–2593.
35. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609–618.
36. Burnett-Hartman AN, Newcomb PA, Potter JD, et al. Genomic aberrations occurring in subsets of serrated colorectal lesions but not conventional adenomas. Cancer Res 2013;73:2863–2872.
37. De Sousa F, Melo E, Wang X, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat Med 2013; 19:614–618.
38. Carragher LA, Snell KR, Giblett SM, et al. V600EBraf induces gastrointestinal crypt senescence and promotes tumour progression through enhanced CpG methylation of p16INK4a. EMBO Mol Med 2010; 2:458–471.
39. Rad R, Cadinanos J, Rad L, et al. A genetic progression model of Braf(V600E)-induced intestinal tumorigenesis reveals targets for therapeutic intervention. Cancer Cell 2013;24:15–29.
40. Andre T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol 2009;27:3109–3116.
41. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. J Clin Oncol 2011;29:3768–3774.
42. Kuebler JP, Wieand HS, O'Connell MJ, et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. J Clin Oncol 2007;25:2198–2204.
43. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol 2010;28:3219–3226.
44. Kang M, Shen XJ, Kim S, et al. Somatic gene mutations in African Americans may predict worse outcomes in colorectal cancer. Cancer Biomark 2013; 13:359–366.
45. Sylvester BE, Huo D, Khramtsov A, et al. Molecular analysis of colorectal tumors within a diverse patient cohort at a single institution. Clin Cancer Res 2012; 18:350–359.
46. Andreyev HJ, Norman AR, Cunningham D, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer 2001;85:692–696.
47. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010;11:753–762.
48. Imamura Y, Morikawa T, Liao X, et al. Specific mutations in KRAS codons 12 and 13, and patient prognosis in 1075 BRAF wild-type colorectal cancers. Clin Cancer Res 2012;18:4753–4763.
49. Farina-Sarasqueta A, van Lijnschoten G, Moerland E, et al. The BRAF V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. Ann Oncol 2010;21:2396–2402.
50. Ogino S, Shima K, Meyerhardt JA, et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803. Clin Cancer Res 2012;18:890–900.
51. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 2012;483:100–103.
52. Corcoran RB, Ebi H, Turke AB, et al. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. Cancer Discov 2012; 2:227–235.

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Conflicts of interest
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