

Prognostic Significance of Defective Mismatch Repair and BRAF V600E in Patients with Colon Cancer

Amy J. French,¹ Daniel J. Sargent,² Lawrence J. Burgart,³ Nathan R. Foster,² Brian F. Kabat,² Richard Goldberg,⁴ Lois Shepherd,⁵ Harold E. Windschitl,⁶ and Stephen N. Thibodeau¹

Abstract Purpose: Colon tumors with defective DNA mismatch repair (dMMR) have a well-characterized phenotype and accounts for ~15% to 20% of sporadic colon cancer as well as those colon cancer patients with Lynch syndrome. Although the presence of dMMR seems to be a favorable prognostic marker, data suggest that these patients do not respond as well to adjuvant chemotherapy.

Experimental Design: In this study, we examined the prognostic significance of tumor MMR deficiency and the presence of a specific mutation in *BRAF* (V600E) in a group of patients ($n = 533$) who participated in a randomized prospective clinical trial through the North Central Cancer Treatment Group.

Results: Tumors with dMMR were found to be associated with higher tumor grade ($P = 0.001$), proximal location ($P < 0.0001$), and improved overall and disease-free survival ($P = 0.05$ and 0.04 , respectively). Among all cases examined, evaluation of the *BRAF* V600E mutation status revealed no statistically significant differences in either disease-free or overall survival. Patients were then grouped into four categories for further analysis: dMMR/*BRAF*(-), dMMR/*BRAF*(+), pMMR/*BRAF*(-), and pMMR/*BRAF*(+). The dMMR/*BRAF*(-) group had a significantly improved overall survival (5-year overall survival of 100% versus 73%, $P = 0.002$) compared with all others. The remaining three groups had very similar survival outcomes. An additional cohort of tumors previously classified as having dMMR were also tested for the *BRAF* V600E alteration. Results remained significant ($P = 0.006$) when the two groups were combined for analysis.

Conclusions: Overall, these data suggest that the underlying molecular etiology of those tumors having dMMR may influence the disease outcome in these patients.

Colon cancer is one of the leading causes of cancer deaths worldwide. It is estimated that more than 148,000 new cases of colon cancer were reported in the United States in 2006 (1). Rather than assuming that colon cancers are homogeneous, an assessment of prognosis based on features of the resected tumor would permit treating physicians to qualify the benefit of adjuvant chemotherapy to individual patients. Currently, anatomic and pathologic staging is still the most accurate predictor of patient outcome. It would be valuable to supplement standard clinical and pathologic staging using molecular markers

to more precisely define the subset of patients at highest or lowest risk of relapse following colon cancer surgery. This would facilitate better selection of high-risk patients (those who would benefit most from adjuvant therapy) and of low-risk patients (those with a lower likelihood of a treatment benefit who would instead potentially benefit from avoiding the toxicity, expense, inconvenience, and risks of adjuvant therapy). One of the most promising molecular markers investigated in colon cancer to date is the presence of tumor microsatellite instability (2).

Based on the presence or absence of functional DNA mismatch repair (MMR), colon cancer is generally divided into two broad categories (3, 4). Tumors with defective MMR (dMMR) are characterized by the presence of a particular tumor phenotype, termed microsatellite instability (MSI), and by the absence of protein expression for any one of a number of genes involved in MMR, including *hMLH1*, *hMSH2*, *hMSH6*, or *PMS2* (5, 6). Tumors with dMMR have been identified in ~15% to 20% of sporadic colon cancer and in patients with Lynch syndrome, a subset of hereditary nonpolyposis colon cancer (7). In sporadic colon cancer, three distinct MSI phenotypes have been described: MSS (none of the examined loci show instability), MSI-L (MSI at <30% of loci examined), and MSI-H (MSI at $\geq 30\%$ of loci examined; ref. 8). The MSI-H phenotype is associated with distinct clinicopathologic features including proximal tumor site, high grade, early stage, and diploidy (9–13). Importantly, this phenotype has been

Authors' Affiliations: ¹Laboratory Medicine and Pathology and ²Health Sciences Research, Mayo Clinic College of Medicine, Rochester, Minnesota; ³Department of Pathology, Abbott Northwestern Hospital/Virginia Piper Cancer Institute, Minneapolis, Minnesota; ⁴Division of Hematology and Oncology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; ⁵National Cancer Institute of Canada Clinical Trials Group, Queen's University, Kingston, Ontario, Canada; and ⁶CentraCare Clinic, St. Cloud, Minnesota

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Requests for reprints: Stephen N. Thibodeau, Mayo Clinic College of Medicine, 200 First Street Southwest, 920 Hilton Building, Rochester, MN 55905. Phone: 507-284-9185; Fax: 507-284-0670; E-mail: sthibodeau@mayo.edu.

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associated with a more favorable outcome (2). Among sporadic colon cancer, the majority of MSI-H cases are due to inactivation of *hMLH1* (~95%), with *hMSH2* and *hMSH6* accounting for a smaller percentage, ~5% and <1%, respectively (14). Germ-line mutations in these same MMR genes are responsible for Lynch syndrome, with *hMLH1* and *hMSH2* accounting for the majority of cases (~40% each) and *hMSH6* and *PMS2* again accounting for a smaller percentage, ~10% and 5%, respectively (15, 16). Among all cases involving *hMSH2* and *hMSH6* (sporadic or inherited), the presence of a germ-line mutation seems to be the most common mechanism of gene inactivation. For *hMLH1*, however, current data suggest that the most common mechanism (~90% of cases) of gene inactivation among unselected cases is promoter hypermethylation and, less frequently, by mutations in the gene itself (14, 17, 18). Thus, the molecular etiology of those tumors involving dMMR is very heterogeneous, involving several different genes and numerous mechanisms of gene inactivation, including epigenetic, somatic, and germ-line alterations.

The *Ras/Raf/MEK/MAP* kinase cascade is an essential component of intracellular signaling from activated cell-surface receptors to transcription factors in the cell nucleus. Mutations of the *Raf* activator *Ras* are present in 30% of human cancers (19, 20) and their transforming potential is dependent on *Raf* (21). *BRAF* is one of three known *Raf* genes thought to have arisen from gene duplication (the other two are *ARAF1* and *CRAF*). Davies et al. (22) reported the presence of *BRAF* somatic mutations in 66% of malignant melanomas and at a lower frequency in a wide range of other human cancers, including colon cancers. *BRAF* mutations in colon cancer were then reported to occur more frequently in those cases characterized by the presence of dMMR (23). Although the etiology is still ill-defined, *BRAF* mutations were found in subsequent studies to occur almost exclusively in tumors showing the involvement of *hMLH1* due to promoter hypermethylation. Current studies suggest that the *BRAF* V600E alteration occurs in ~10% to 15% of tumors that are proficient in the MMR pathway (MSS/MSI-L) and in ~70% of tumors that have dMMR (MSI-H) due to promoter hypermethylation of *hMLH1*. *BRAF* mutations rarely, if ever, occur in tumors with dMMR due to the presence of germ-line mutations (24, 25). Thus, *BRAF* V600E is tightly associated with dMMR due to *hMLH1* promoter hypermethylation but not with dMMR due to germ-line alterations.

Given that the survival advantage reported in dMMR tumors is represented mostly by tumors with *hMLH1* promoter hypermethylation, the V600E mutation may provide additional predictive value about disease outcome. In this study, we sought to determine if the *BRAF* V600E alteration, in addition to the MMR status of the tumor, was able to better define patient outcome. For this analysis, we examined 533 tumors from patients classified as either high-risk stage II or stage III colon cancer who participated in a randomized prospective clinical trial. The MMR status was defined by both immunohistochemical staining of paraffin tissue coupled with testing for MSI. Tumor was then examined for the V600E mutation within the *BRAF* gene.

Materials and Methods

Patient population. Paraffin-embedded tissues were obtained from patients enrolled in a phase III randomized trial (Intergroup 0135/NCCTG 91-46-53/NCIC CTG CO.9), which evaluated high-dose versus

standard-dose levamisole when combined with 5-fluorouracil and leucovorin as adjuvant therapy following en bloc resection of colon cancer. To be enrolled in the clinical trial, patients were required to be at high risk for tumor recurrence following surgery, as indicated by one or more of the following features of the primary colon cancer: (a) regional lymph node metastases; (b) transmural tumor involvement through the serosa; (c) pericolic fat invasion and tumor perforation, adherence to, or invasion of adjacent organ(s); or (d) central laboratory flow cytometry determination of either nondiploid or proliferation index (%S phase + %G₂-M) >0.20 (26). Patients with stage II lesions were eligible if associated with any of the two criteria noted above. Patients with distant metastases were excluded. A total of 878 patients were enrolled in this trial. At the time the molecular study was conducted, tissue blocks were available from 562 patients, 533 (95%) of which had a sufficient amount of tumor for analysis. This sample represents a nonrandom subset of the population from which it was drawn. This molecular correlative study was reviewed and approved by local institutional review boards.

In an independent validation set, paraffin-embedded tumors that had previously been characterized as having dMMR were obtained for further analysis (27). This prior study included patients from seven different North Central Cancer Treatment Group Phase III clinical trials investigating surgical adjuvant chemotherapy in stage II and stage III patients having en bloc resection of either colon or rectal carcinoma as previously described. For details of treatments used and follow-up protocol, see Halling et al. (27).

Tissue selection and DNA extraction. Paraffin blocks were serially cut into 5- or 10- μ m-thick sections. Slide 6 (of 20) was stained with H&E and areas of neoplastic (>50%) and normal tissue were identified by a pathologist. The areas containing marked normal and tumor tissue from slides 1 through 5 were scraped and placed into separate tubes for DNA extraction using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions.

Immunohistochemistry. Slides 7 through 9 were selected for immunohistochemical staining with antibodies to *hMLH1*, *hMSH2*, and *hMSH6* as previously described (28). When needed, additional testing for *PMS2* protein expression was done as previously described (29).

MSI testing. PCR for the various microsatellite markers was carried out on matched tumor and normal DNA for each of the patients studied. Standard PCR conditions were used and included 10 \times buffer type II, Taq gold, and deoxynucleotide triphosphates. Primers were custom ordered with various fluorescent dyes from Applied Biosystems. PCR product was analyzed on an ABI 3100 (Applied Biosystems).

Mismatch repair definition. Defective mismatch repair (dMMR) was defined by the presence of MSI at the marker BAT 26 coupled with absence of protein expression for *hMLH1*, *hMSH2*, or *hMSH6*. For patients that were stable at BAT 26 and showed normal protein expression for all three proteins, 16 additional microsatellite markers (*myc-L*, *D8S262*, *D8S1742*, *D8S261*, *D8S254*, *D8S133*, *D8S136*, *D8S560*, *D8S1055*, *D8S1820*, *D8S255*, *ANK1*, *D8S1760*, *D8S1720*, *D8S1842*, and *D8S1925*) were used to test for MSI. If >30% of the markers showed MSI, then that tumor was classified as MSI-H and additional immunohistochemistry testing was done with antibody for *PMS2*. These were also defined as having dMMR.

BRAF testing. Testing for the *BRAF* V600E mutation in exon 15 was done by conformation-sensitive gel electrophoresis (CSGE) analysis (30), DNA sequencing, or both for the 533 tumors that were entered onto this study, regardless of the MMR status. PCR primers for this assay (available on request) were custom ordered from Integrated DNA Technologies. Standard PCR conditions were used and included 10 \times buffer type II, Taq gold, and deoxynucleotide triphosphates from Applied Biosystems. For the CSGE analysis, PCR product was denatured at 95°C for 5 min and cooled to 65°C over 30 min. The reannealed product was mixed with loading dye (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF) and then loaded onto a CSGE gel consisting of 15% acrylamide/1,4-bis(acryloyl)piperazine (19:1), 0.5 \times TTE buffer (44.4 mmol/L Tris, 14.25 mmol/L Taurine, and

0.1 mmol/L EDTA, pH 9.0), 15% formamide, and 10% ethylene glycol. The gel was run at 30 W for 5 h. DNA sequence analysis was done on all samples that showed an abnormally migrating fragment by CSGE, all ambiguous CSGE results, and all cases that were classified as having dMMR tumors by MSI and immunohistochemistry testing of tumor tissue.

Statistical methods. Overall survival (OS; censored at 8 y) was calculated as the number of days from random assignment to the date of death or last contact. Disease-free survival (DFS; censored at 5 y) was calculated as the number of days from random assignment to the date of disease recurrence or death. The distributions of OS and DFS were estimated using the Kaplan-Meier method (31). Univariate and multivariate Cox proportional hazards models were used to explore the association of clinical and laboratory parameters with OS and DFS (32). The score statistic was used to test for significance in univariate models. The likelihood ratio test was used to test for the significance of a single covariate in the presence of, or adjusting for, other covariate(s) in multivariate models.

Laboratory parameters (e.g., MMR status, *BRAF*) were correlated with clinical characteristics (e.g., stage, grade). Consistent with previous practice (27), MSI-L and MSS patients were grouped together and classified as proficient MMR (pMMR) for the purposes of this analysis. Pooling of this data was confirmed in this data set as there were no statistically significant differences between the MSI-L and MSS patient groups for OS or DFS. Summary statistics (e.g., mean, median) and frequency tables were used to describe the distributions of parameters investigated. Appropriate statistical tests were used to test for differences in the distributions of continuous and categorical variables (e.g., *t* test, Wilcoxon, χ^2 , Fisher's exact tests). All statistical tests were two sided and $P \leq 0.05$ was considered statistically significant. *P* values were not adjusted for multiple comparisons. Statistical analyses were done via SAS software (SAS Institute).

Results

Of the 562 patients enrolled in this study, 17 were eliminated due to the absence of usable tumor in available tissue. An additional 12 patients had tumor present, but the tumor percentage was lower than the established threshold for reliable MSI testing. The remaining 533 patients were evaluated by immunohistochemistry for the presence or absence of hMLH1, hMSH2, and hMSH6 and for MSI with the mononucleotide repeat marker *BAT 26*. Fifty-seven cases showed both an absence of protein expression for at least one of the MMR genes and showed MSI at *BAT 26*, and these were classified as having dMMR. Of the 57 cases with dMMR, 44 had an absence of protein expression for hMLH1, 3 for hMLH1/hMSH6, 9 for hMSH2/hMSH6, and 1 for hMSH6 alone. Four additional cases with dMMR were also identified. Two of these each had an absence of protein expression for one of the MMR genes (one for hMLH1 and one for hMSH2/hSMH6), but due to poor quality DNA in the extracted specimen or a lack of sufficient tumor area for microdissection, no MSI testing was possible. These two patients were used for the MMR analyses, but were not included in the *BRAF* portion of this study because no DNA was available. In a third case, tumor was positive for instability at *BAT 26*, but had normal expression for all three proteins. After additional testing, this case was shown to have an absence of PMS2 staining. The final case showed normal protein expression for all four MMR proteins tested, was negative for *BAT 26* instability, but subsequently showed instability at 15 of 16 (94%) microsatellite markers that were additionally tested. Thus, 61 (11%) cases were classified as having dMMR and 472 (89%) were classified as having pMMR.

Patient and tumor characteristics with respect to the MMR status are shown in Table 1. Overall, tumors with dMMR were more likely to be high grade ($P = 0.001$) and more frequently located on the proximal side of the colon ($P < 0.0001$; Table 1). There were no statistically significant differences in age, gender, or stage.

Testing for the V600E mutation in the *BRAF* gene was done on tumor DNA from 533 patients but informative results were obtained for only 490. The DNA from the missing 43 samples was not of sufficient quality for the CSGE and DNA sequence analyses. Patient and tumor characteristics with respect to both the MMR and *BRAF* V600E status are shown in Table 2. Of the 490 cases where both the MMR and *BRAF* mutation status was defined, 23 (4.7%) cases with dMMR were *BRAF*(-) (9 with loss of hMSH2/hMSH6, 1 for hMSH6 alone and 1 from PMS2 alone) and 35 (7.1%) were *BRAF*(+) (all demonstrating loss of hMLH1), and 390 (79.6%) were *BRAF*(+), whereas 390 (79.6%) cases with pMMR were *BRAF*(-) and 42 (8.6%) were *BRAF*(+). Among these four groups, significant differences were observed for age [*BRAF*(-)/dMMR cases more likely to have a younger age of diagnosis, $P = 0.0015$], gender [*BRAF*(+)/dMMR cases more likely to be female, $P = 0.0204$], grade [*BRAF*(-)/pMMR cases more likely to have lower grade disease, $P < 0.0001$], and site [*BRAF*(-) and *BRAF*(+)/dMMR cases more likely to be located in the proximal colon, $P < 0.0001$]. Overall, no differences were noted in the stage distribution among these four groups. The fraction of stage II cases in all groups was ~25%.

In univariate survival analysis (Table 3), significantly improved DFS ($P < 0.001$) and OS ($P < 0.001$) were observed for stage (II versus III). In addition, dMMR patients were found to have significantly improved DFS ($P = 0.04$) and OS ($P = 0.05$) compared with pMMR patients. For the MMR status, the DFS and OS rates were 82% and 87%, respectively, in patients whose tumors exhibited dMMR, compared with 67% and 72%, respectively, in patients with pMMR tumors. This improved DFS and OS did not depend on stage or grade ($P_{\text{interaction}} \geq 0.50$). Finally, no significant differences were found with respect to *BRAF* mutations for either DFS or OS when the entire population was evaluated ($P \geq 0.31$; Table 3).

Additional analyses were then done taking into account both the MMR and *BRAF* status as shown in Table 2. Of the four groups examined, patients with dMMR/*BRAF*(-) tumors had significantly improved OS ($P = 0.002$; Fig. 1B; Table 3), but not DFS ($P = 0.06$; Fig. 1A; Table 3), compared with the other three patient groups combined. In addition, within the subgroup of patients with dMMR tumors, the 23 patients with *BRAF*(-) tumors had significantly better OS than the 35 *BRAF*(+) patients (100% versus 77%, $P = 0.001$); no difference was observed by *BRAF* status in the 432 pMMR patients for DFS or OS ($P \geq 0.57$). Thus, the *BRAF* mutation status seems to be important in the group of cases having dMMR but not in the group of patients whose tumors had pMMR. The lack of deaths in the dMMR/*BRAF*(-) group prevented any multivariate modeling to adjust for other covariates including age. However, we completed a sensitivity analysis for OS and found that the results remained significant for dMMR/*BRAF*(-) patients versus all others and versus only the dMMR/*BRAF*(+) patients, even after we used a model that presumed up to three hypothetical patient deaths in this cohort, both in univariate models ($P = 0.042$) and in limited multivariate models adjusting individually for age ($P = 0.03$), stage ($P = 0.01$),

Table 1. Patient characteristics by MMR status

| | pMMR (n = 472; 88.6%) | dMMR (n = 61; 11.4%) | Total (n = 533) | P |
|---------------|-----------------------|----------------------|-----------------|-----------|
| Age, y | | | | |
| Mean (SD) | 61.5 (10.21) | 60.9 (11.77) | 61.5 (10.39) | 0.84* |
| Median | 63.0 | 64.0 | 63.0 | |
| Range | (31.0-85.0) | (30.0-78.0) | (30.0-85.0) | |
| Gender, n (%) | | | | |
| F | 220 (46.6) | 35 (57.4) | 255 (47.8) | 0.11 † |
| M | 252 (53.4) | 26 (42.6) | 278 (52.2) | |
| Grade, n (%) | | | | |
| 1 and 2 | 344 (72.9) | 32 (52.5) | 376 (70.5) | 0.001 † |
| 3 and 4 | 128 (27.1) | 29 (47.5) | 157 (29.5) | |
| Site, n (%) | | | | |
| Proximal | 205 (43.6) | 53 (86.9) | 258 (48.6) | <0.0001 † |
| Distal | 265 (56.4) | 8 (13.1) | 273 (51.4) | |
| Stage, n (%) | | | | |
| II | 125 (26.5) | 15 (24.6) | 140 (26.3) | 0.75 † |
| III | 347 (73.5) | 46 (75.4) | 393 (73.7) | |

*Wilcoxon rank-sum P value.
† χ^2 P value.

and tumor grade ($P = 0.01$). Although we did not censor deaths as part of the statistical analysis, a review of the medical records was done to determine cause of death: eight subjects died of cancer, four subjects died of other causes (no evidence of disease), and in one case the cause of death was not documented.

Overall, our results suggest that the *BRAF* mutation status may provide additional prognostic information among those cases that have dMMR. Because of the limited sample size in the initial analysis, however, *BRAF* testing was done on a second set of cases ($n = 76$) previously identified with dMMR (27). Of the 76 patients, 48 were found to be positive for the *BRAF* V600E alteration. Although no statistically significant differences in DFS and OS were observed between tumors with and without *BRAF* mutations individually in this validation cohort, the results showed a trend toward improved survival for the *BRAF*(-) group, similar to that found in the initial

analysis. When both groups of patients were combined ($n = 134$), a significant difference in OS was retained ($P = 0.006$), with patients with dMMR tumors without a *BRAF* mutation showing improved survival compared with those with a *BRAF* mutation (Fig. 2B; Table 4).

Discussion

The presence of dMMR, as assessed by the presence of tumor MSI (specifically the MSI-H phenotype), continues to be one of the most consistent and promising molecular markers of prognosis for colon cancer (2). In this study, we show that dMMR (MSI-H phenotype) provides prognostic information with a magnitude of effect consistent with that previously observed (~15% increase in 5-year DFS and OS; $P = 0.04$ and $P = 0.05$, respectively; refs. 2, 27, 33). Furthermore, this phe-

Table 2. Patient characteristics by *BRAF*/MMR

| | <i>BRAF</i> (-)/dMMR (n = 23; 4.7%) | <i>BRAF</i> (-)/pMMR (n = 390; 79.6%) | <i>BRAF</i> (+)/dMMR (n = 35; 7.1%) | <i>BRAF</i> (+)/pMMR (n = 42; 8.6%) | Total (n = 490) | P |
|---------------|--|--|--|--|-----------------|-----------|
| Age, y | | | | | | |
| Mean (SD) | 53.8 (12.69) | 61.5 (10.12) | 65.9 (7.29) | 60.5 (11.49) | 61.4 (10.38) | 0.0015* |
| Median | 52.0 | 63.0 | 67.0 | 59.5 | 63.0 | |
| Range | (30.0-72.0) | (31.0-85.0) | (50.0-78.0) | (33.0-83.0) | (30.0-85.0) | |
| Gender, n (%) | | | | | | |
| F | 8 (34.8) | 185 (47.4) | 25 (71.4) | 18 (42.9) | 236 (48.2) | 0.02 † |
| M | 15 (65.2) | 205 (52.6) | 10 (28.6) | 24 (57.1) | 254 (51.8) | |
| Grade, n (%) | | | | | | |
| 1 and 2 | 12 (52.2) | 299 (76.7) | 17 (48.6) | 23 (54.8) | 351 (71.6) | <0.0001 † |
| 3 and 4 | 11 (47.8) | 91 (23.3) | 18 (51.4) | 19 (45.2) | 139 (28.4) | |
| Site, n (%) | | | | | | |
| Proximal | 17 (73.9) | 162 (41.8) | 33 (94.3) | 28 (66.7) | 240 (49.2) | <0.0001 † |
| Distal | 6 (26.1) | 226 (58.2) | 2 (5.7) | 14 (33.3) | 248 (50.8) | |
| Stage, n (%) | | | | | | |
| II | 6 (26.1) | 108 (27.7) | 8 (22.9) | 11 (26.2) | 133 (27.1) | 0.94 † |
| III | 17 (73.9) | 282 (72.3) | 27 (77.1) | 31 (73.8) | 357 (72.9) | |

*Kruskal-Wallis rank-sum P value.
† χ^2 P value.

Table 3. Five-year survival rates and univariate associations with survival

| Factor | n = 533 | 5-y DFS (%) | HR (95% CI) | P* | 5-y OS (%) | HR (95% CI) | P* |
|---------------|---------|-------------|-----------------|--------|------------|---|---------|
| Age | | | | | | | |
| 10-y increase | 533 | | 1.008 (0.9-1.2) | 0.92 | | 1.1 (0.98-1.3) | 0.09 |
| Stage | | | | | | | |
| II | 140 | 83 | 2.4 (1.5-3.7) | <0.001 | 87 | 2.2 (1.4-3.2) | <0.001 |
| III | 393 | 64 | | | 69 | | |
| Grade | | | | | | | |
| 1 and 2 | 376 | 70 | 1.2 (.9-1.7) | 0.28 | 77 | 1.3 (1.0-1.8) | 0.06 |
| 3 and 4 | 157 | 66 | | | 68 | | |
| Site | | | | | | | |
| Proximal | 258 | 69 | 1.0 (.7-1.3) | 0.90 | 72 | 1.1 (0.8-1.5) | 0.51 |
| Distal | 273 | 68 | | | 76 | | |
| Gender | | | | | | | |
| Female | 255 | 71 | 1.2 (0.9-1.6) | 0.25 | 76 | 1.2 (0.9-1.6) | 0.26 |
| Male | 278 | 67 | | | 72 | | |
| MMR | | | | | | | |
| pMMR | 472 | 67 | 0.5 (0.3-1.0) | 0.04 | 72 | 0.6 (0.3-1.0) | 0.05 |
| dMMR | 61 | 82 | | | 87 | | |
| BRAF | | | | | | | |
| Negative | 413 | 68 | 1.0 (0.6-1.6) | 0.97 | 75 | 1.2 (.8-1.8) | 0.31 |
| Positive | 77 | 70 | | | 71 | | |
| pMMR | | | | | | | |
| BRAF(-) | 390 | 67 | 1.2 (0.7-2.0) | 0.57 | 73 | 1.2 (0.7-1.9) | 0.59 |
| BRAF(+) | 42 | 64 | | | 67 | | |
| dMMR | | | | | | | |
| BRAF(-) | 23 | 87 | 2.0 (0.5-7.5) | 0.30 | 100 | Cox model inappropriate due to lack of events | 0.001 † |
| BRAF(+) | 35 | 77 | | | 77 | | |
| BRAF and MMR | | | | | | | |
| BRAF(-)/dMMR | 23 | 87 | 0.4 (0.1-1.1) | 0.06 | 100 | Cox model inappropriate due to lack of events | 0.002 † |
| Other | 467 | 68 | | | 73 | | |

*Univariate score statistic.

†Log-rank P: no hazard ratio due to shortage of events.

notype may also be important in predicting treatment effects (33). Ribic et al. (33) suggested that patients whose tumors are characterized by the presence of dMMR may be adversely effected when treated with a 5-fluorouracil-based regimen. However, given that the molecular etiology of those tumors involving dMMR is very heterogeneous (different genes and different mechanisms of gene inactivation—epigenetic, somatic, and germ-line alterations), it is very likely to be the case that not all of these tumors will behave in a similar fashion.

The presence of tumor heterogeneity led us to explore the utility of additional markers, specifically, the presence or absence of the V600E alteration in the BRAF proto-oncogene. As noted earlier, BRAF V600E is tightly associated with dMMR due to hMLH1 promoter hypermethylation but not with dMMR due to germ-line alterations. The BRAF V600E alteration occurs in ~10% of tumors that are MSS/MSI-L and in ~70% of tumors that are MSI-H with hMLH1 promoter hypermethylation. BRAF mutations have not been found in tumors that are MSI-H due to the presence of germ-line mutations in hMLH1, hMSH2, or hMSH6 (24, 25). Additionally, both hMLH1 methylation-associated MSI and the presence of the BRAF V600E alteration have also been strongly associated with tumors that have the CpG island methylator phenotype (CIMP; refs. 24, 25, 34, 35). Because hMLH1 methylation-associated MSI generally does not occur among sporadic cases outside the context of CIMP, it seems that the underlying basis for MMR deficiency among this select group of sporadic colon cancer is a broader epigenetic control defect that affects hMLH1 in some, but not

all, CIMP tumors. Furthermore, the CIMP phenotype seems to also underlie nearly all tumors characterized by the presence of the BRAF V600E alteration. Cumulatively, these data highlight the heterogeneity of colon cancers. Understanding these differences will be critical for optimal patient care, and the BRAF V600E may provide a useful marker representing a unique subset of colon cancer.

With this information in mind, the clinical significance of the BRAF V600E alteration was examined across the entire group of patients and among subsets defined by the MMR status (Table 2). Among the groups examined, BRAF V600E provided additional prognostic information only for the subset of patients having dMMR. Among patients with dMMR, BRAF(-) cases had a significantly improved OS compared with those that were BRAF(+) (P = 0.001). There were no differences in DFS or OS among cases with pMMR tumors (P = 0.57 and P = 0.59, respectively). When all four groups were compared, patients whose tumors were BRAF(-)/dMMR continued to show significantly improved OS (P = 0.002).

Because the sample size among the various subsets with dMMR was small, we identified an additional cohort of patients with known dMMR status as a second validation set (27). When examined, the results of the BRAF testing showed a similar trend in OS, but the results were not statistically significant. There may be a number of reasons for this difference. First, the average age of patients with dMMR within the validation cohort is older (median age, 67.5 years; range, 39-86 years) compared with the original cohort (median age, 63.0 years;

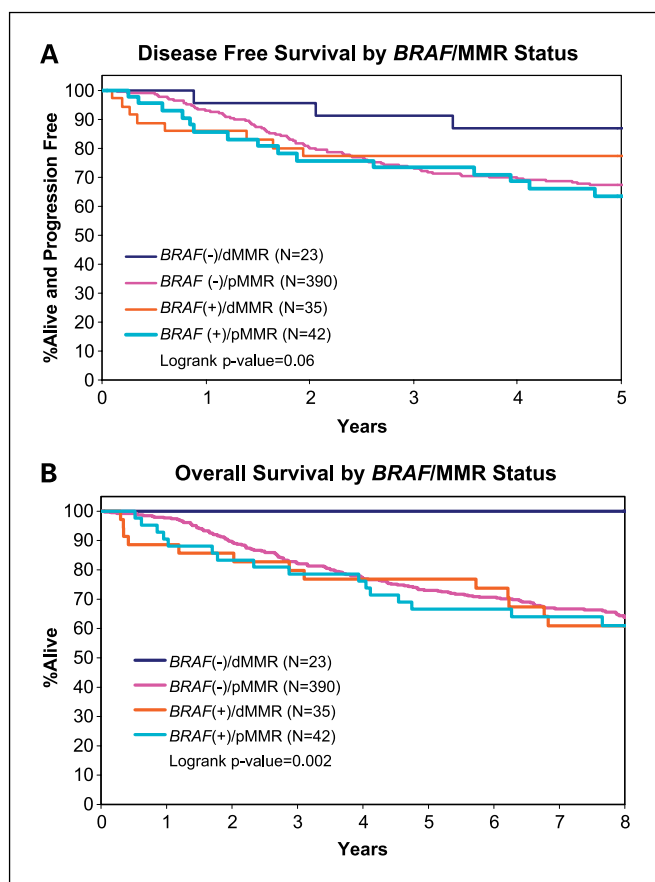


Fig. 1. Kaplan-Meier plots for the four *BRAF*/*MMR* subgroups. The log-rank *P* value is derived by comparing DFS (A) or OS (B) for all four patient groups.

range, 30-85 years), suggesting a possible difference in the proportion of germ-line versus sporadic cases within each group. Second, the validation cohort of patients was obtained from several different clinical trials that accrued from 1978 to 1989. The treatment and care of patients has improved over the years, potentially affecting our ability to observe a *BRAF* effect in earlier trials. Surgical techniques have also improved over the last 20 years, which might affect survival; descriptively comparing outcomes of stage-matched patients, our more recent cohort had improved OS compared with the previous cohort (5-year OS was 87% and 69% for stage II and stage III patients, respectively, compared with rates of 81% and 58%, respectively, in the previous cohort). The most likely explanation, however, is that our original group of patients with dMMR likely overrepresent cases for improved survival and the previous group might underrepresent cases for improved survival. On combining the two studies, the *BRAF* status among patients with dMMR was still significantly associated with OS, with improved survival in the dMMR/*BRAF*(-) group ($P = 0.006$).

The clinical significance of *BRAF* V600E in colon cancer has been examined in only one other study (36). In contrast to our current study, Samowitz et al. showed that microsatellite-unstable tumors (dMMR) were associated with an excellent 5-year survival regardless of the V600E status. However, these investigators also report that the V600E mutation was associated with poor survival among the microsatellite-stable

tumors (pMMR; ref. 36). We found no difference in this subset of patients. Although the discrepancies between these two studies are perplexing, there are some differences in the two study populations. Our study was confined to high-risk stage II and stage III disease, whereas all four stages were represented in the study by Samowitz et al. The number of *BRAF*(+) cases among the pMMR cases in the series reported by Samowitz was approximately half of what was observed in this study [40 of 803 (5%) versus 42 of 432 (10%), respectively]. This apparent difference may again be due to the stage differences between the two studies. In the study reported by Samowitz et al. (36), *BRAF* V600E was found to be highly correlated with stage, with higher frequency of this mutation found in cases with higher stage. The presence of stage I and low-risk stage II cases in the study by Samowitz et al. would dilute the overall number of cases showing the *BRAF* alteration (they report only 7 mutations among 400 cases in these two groups). Finally, participants of the study by Samowitz et al. represented different racial groups and they were not in a controlled clinical trial setting. Because there are differences between these studies, it is difficult to compare results directly. Clearly, additional studies with larger patient groups are warranted.

Among our cases with *BRAF*(-)/dMMR tumors ($n = 23$), the age at diagnosis was nearly 10 years younger than the

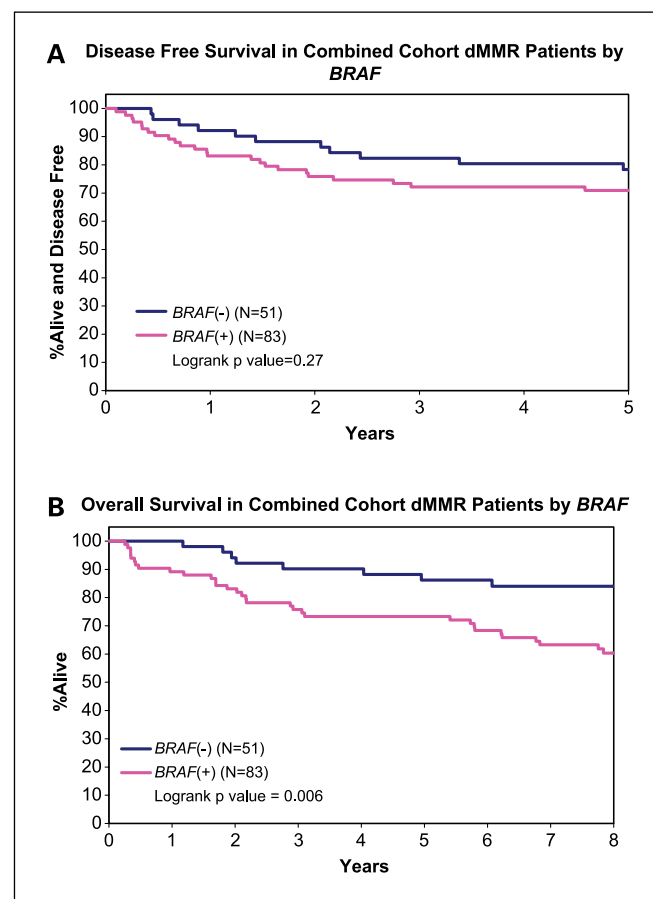


Fig. 2. Kaplan-Meier plots for the combined cohort of dMMR patients by *BRAF*. The log-rank *P* value is derived by comparing DFS (A) or OS (B) for the two patient groups.

Table 4. Univariate analysis in combined cohort

| Factor | n = 134* | 5-y DFS (%) | HR (95% CI) | P | 5-y OS (%) | HR (95% CI) | P |
|-----------------|----------|-------------|---------------|------|------------|---------------|-------|
| dMMR tumors | | | | | | | |
| <i>BRAF</i> (-) | 51 | 78 | 1.5 (0.7-3.1) | 0.27 | 86 | 2.9 (1.3-6.4) | 0.006 |
| <i>BRAF</i> (+) | 83 | 71 | | | 73 | | |

NOTE: Score *P* value was computed after stratifying by study.
*Three patients are missing *BRAF* information.

other three groups (median age, 52 years; range, 30-72 years; $P = 0.0015$). This data suggests that this group is composed of a higher fraction of germ-line cases. This is consistent with both the fact that germ-line cases (Lynch syndrome) have a younger age of onset (37) and that *BRAF* mutations are associated with hypermethylation of the *hMLH1* promoter but not germ-line derived dMMR (24, 25, 34, 38, 39). Of the 10 patients that had an absence of protein expression for either hMSH2 ($n = 9$) or hMSH6 ($n = 1$), all were negative for a *BRAF* mutation. Absence of hMSH2 or hMSH6 is almost always associated with a germ-line event. Of the 12 that have an absence of protein expression for hMLH1 in the *BRAF*(-)/dMMR group, some of these are also likely to be germ-line. Given the young age at diagnosis for a substantial fraction of the *BRAF*(-)/dMMR cases, the role of age in our findings must be considered. Survival may be different between the two groups for a variety of reasons. Older subjects may have more comorbidity and may be more likely to die from unrelated causes. Additionally, it may be that the adverse effects of chemotherapy described by Ribic et al. (33) may apply to subjects with sporadic MSI-H colorectal cancer but not to those with hereditary nonpolyposis colon cancer (who may benefit from chemotherapy). At this point, there is essentially no data in the literature that helps to address these issues. Fortunately for the patients, but unfortunately for our analysis, the complete absence of any deaths in this group of patients prohibited multivariate modeling to adjust for age.

However, our sensitivity analyses, where three hypothetical events were added to the *BRAF*(-)/dMMR patient group, suggest that these results would be maintained were a true multivariate analysis possible.

Colon cancer with dMMR (MSI-H) is recognized to be heterogeneous. Thus, even for this rather defined group, it would still be valuable to identify additional prognostic markers. Given that *BRAF* V600E mutation occurs at a high frequency in this group and has the ability to help distinguish inherited from sporadic cases, this marker provides a potential approach to more precisely define the subset of patients at highest or lowest risk of relapse following colon cancer surgery. Overall, our study showed that the concomitant evaluation of both the MMR status and *BRAF* mutation status in colon cancer provides useful prognostic information beyond evaluation of either factor alone. Patients in whose tumors are *BRAF*(-)/dMMR have a significantly improved OS. However, given the difficulty in obtaining a sufficiently large number of patients with this tumor phenotype, additional confirmatory studies pooling specimens and data from multiple trials will be required to confirm these findings. It seems likely that over time, with further research, our understanding of the taxonomy of colon cancer will become clearer and more complex as we unravel the important molecular markers of tumor heterogeneity and the relevance of these markers to patient management.

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