# Poor Survival Associated with the *BRAF* V600E Mutation in Microsatellite-Stable Colon Cancers

Wade S. Samowitz,[1] Carol Sweeney,[2] Jennifer Herrick,[2] Hans Albertsen,[2] Theodore R. Levin,[3] Maureen A. Murtaugh,[2] Roger K. Wolff,[2] and Martha L. Slattery[2]

[1]Department of Pathology, University of Utah Health Sciences Center; [2]Department of Family and Preventive Medicine, Health Research Center, Salt Lake City, Utah and [3]Kaiser Permanente Medical Center, Walnut Creek, California

## Abstract

**The *BRAF* V600E mutation has been associated with microsatellite instability and the CpG island methylator phenotype (CIMP) in colon cancer. We evaluated a large population-based sample of individuals with colon cancer to determine its relationship to survival and other clinicopathologic variables. The V600E *BRAF* mutation was seen in 5% (40 of 803) of microsatellite-stable tumors and 51.8% (43 of 83) of microsatellite-unstable tumors. In microsatellite-stable tumors, this mutation was related to poor survival, CIMP high, advanced American Joint Committee on Cancer (AJCC) stage, and family history of colorectal cancer [odds ratio, 4.23; 95% confidence interval (95% CI), 1.65-10.84]. The poor survival was observed in a univariate analysis of 5-year survival (16.7% versus 60.0%; $P < 0.01$); in an analysis adjusted for age, stage, and tumor site [hazard rate ratio (HRR), 2.97; 95% CI, 2.05-4.32]; in stage-specific, age-adjusted analyses for AJCC stages 2 to 4 (HRR, 4.88, 3.60, and 2.04, respectively); and in Kaplan-Meier survival estimates for AJCC stages 2 to 4 ($P < 0.01$ for all three stages). Microsatellite-unstable tumors were associated with an excellent 5-year survival whether the V600E mutation was present or absent (76.2% and 75.0%, respectively). We conclude that the *BRAF* V600E mutation in microsatellite-stable colon cancer is associated with a significantly poorer survival in stages 2 to 4 colon cancer but has no effect on the excellent prognosis of microsatellite-unstable tumors.** (Cancer Res 2005; 65(14): 6063-9)

## Introduction

*BRAF* is a part of the *Ras/Raf/MEK/MAP* signal transduction pathway, and oncogenic mutations in *BRAF*, nearly all of which are the V600E mutation, have been reported in colon cancer (1, 2). We and others have previously observed this mutation in over half of all microsatellite-unstable carcinomas and in a much smaller subset of stable colon tumors (2, 3).[4] In both stable and unstable cancers, >90% of tumors with *BRAF* mutations have widespread methylation of CpG islands or what is known as the CpG island methylator phenotype (CIMP; ref. 3).[4] We and others have also previously reported an improved survival associated with microsatellite instability (MSI) in sporadic colon cancers (4, 5), and because sporadic unstable tumors commonly show both CIMP (6, 7) and *BRAF* mutations (2, 3, 8, 9), one would expect that these features would also show a relationship to improved survival in unstable tumors. The relationship between CIMP and survival in microsatellite-stable tumors has not been extensively studied, however, and to this date, no study has evaluated the relationship between *BRAF* mutations and survival in microsatellite-stable colon cancers. Clinicopathologic associations of the V600E *BRAF* mutation, other than its relationship to MSI and CIMP, have also not been explored.

We have previously used a large population-based series of colon cancers to show numerous relationships between CIMP and clinicopathologic variables, especially the V600E *BRAF* mutation, in multivariate analyses.[4] We now evaluate the relationship between the V600E *BRAF* mutation and/or CIMP and survival in colon cancer as well as the relationship of this mutation to other clinicopathologic variables.

## Materials and Methods

**Study population.** Study participants were White, Black, or Hispanic and were from either the Kaiser Permanente Medical Care Program (KPMCP) of northern California or an eight-county area in Utah (Davis, Salt Lake, Utah, Weber, Wasatch, Tooele, Morgan, and Summit counties). Eligibility criteria for cases included diagnosis with first-primary incident colon cancer (*International Classification of Diseases for Oncology, Second Edition* codes 18.0 and 18.2-18.9) between October 1, 1991 and September 30, 1994 and ages between 30 and 79 years at time of diagnosis. Cases with cancers of the rectosigmoid junction or rectum (defined as the first 15 cm from the anal opening) or with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. All cases were adenocarcinomas or carcinomas. This study population is part of a previously described sample (10). Tumor blocks and amplifiable DNA was originally available on 1,530 individuals, and sufficient DNA for determination of the *BRAF* V600E mutation was available on tumors from 911 individuals. This group did not differ from the original sample with respect to age, gender, American Joint Committee on Cancer (AJCC) stage, histologic differentiation, tumor site, prognosis, or family history of colorectal cancer.

Information on age at time of diagnosis, sex, tumor site, and tumor stage were available from the Northern California Tumor Registry, Sacramento Tumor Registry, and Utah Cancer Registry. These registries are members of the Surveillance, Epidemiology, and End Results program. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexure, descending, and sigmoid colon were defined as distal. Tumors were staged by AJCC criteria (11). Vital status, date of death, primary cause of death, and two contributing causes of death were obtained from local tumor registries using death certificate information. Active follow-up of people diagnosed with cancer is done through the cancer registries on a continuous basis. Vital status as of December 30, 1998 was obtained for all study participants. For individuals whose vital status or cause of death could not be determined through local tumor registries, National Death Index tapes were used. Months of survival were calculated by subtracting the date of last contact or death from the date of diagnosis. Deaths from

**Requests for reprints:** Wade S. Samowitz, Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT 84132. Phone: 801-585-2556; Fax: 801-585-3831; E-mail: wade.samowitz@hsc.utah.edu.



[4] W. Samowitz et al. Multivariate analyses of a large, population-based sample support a CpG island methylator phenotype in colon cancer. Gastroenterol. In press, 2005.

any cause as well as deaths attributed to colon cancer were assessed. Family history of cancer information was collected as part of an interviewer-administered questionnaire. Study participants were asked to list first names of all first-degree blood relatives, including parents, siblings, and children. For each family member enumerated, it was determined if that person ever had been diagnosed with cancer, age at diagnosis, and type of cancer. All aspects of this study were approved by the University of Utah and KPMCP institutional review boards.

***BRAF* V600E mutation detection.** The *BRAF* V600E mutations have been determined in a previous study.[4] Briefly, exon 15 of *BRAF* was amplified from DNA previously extracted from microdissected tumors (4) using the forward primer 5′-TCATAATGCTTGCTCTGATAGGA-3′ and the reverse primer 5′-CTTTCTAGTAACTCAGCAGC-3′. Amplifications were carried out using AmpliTaq Gold and a PCR profile consisting of a 9-minute initial denaturation at 95°C followed by 35 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C with a 5-minute final extension at 72°C. Mutations were verified by sequencing in both directions. Sufficient DNA was available for analysis of 919 tumors. Mutations other than the V600E mutation were identified in 4 tumors but were not used in any of the statistical analyses.

**CpG island methylator phenotype.** CIMP had been determined in a previous study.[4] Briefly, sodium bisulfate modification was done on

**Table 1.** Comparison of *BRAF* V600E mutated to *BRAF* WT in microsatellite-stable and microsatellite-unstable colon cancers with respect to clinicopathologic variables and tumor mutations

| | Stable | | | Unstable | | |
|---|---|---|---|---|---|---|
| | *BRAF* WT, *n* (%) | *BRAF* mutant, *n* (%) | OR* (95% CI) | *BRAF* WT, *n* (%) | *BRAF* mutant, *n* (%) | OR* (95% CI) |
| Tumor site | | | | | | |
| Proximal | 328 (44.4) | 27 (69.2) | 1.00 (Reference) | 33 (82.5) | 37 (92.5) | 1.00 (Reference) |
| Distal | 410 (55.6) | 12 (30.8) | 0.36 (0.18-0.71) | 7 (17.5) | 3 (7.5) | 0.38 (0.09-1.60) |
| AJCC stage† | | | | | | |
| 1 | 192 (25.5) | 1 (2.5) | | 8 (20.0) | 8 (19.0) | 1.00 (Reference) |
| 2 | 208 (27.6) | 6 (15.0) | 1.00 (Reference) | 19 (47.5) | 12 (28.6) | 0.63 (0.19-2.13) |
| 3 | 214 (28.4) | 19 (47.5) | 5.07 (2.10-12.26) | 11 (27.5) | 19 (45.2) | 1.73 (0.51-5.91) |
| 4 | 139 (18.5) | 14 (35.0) | 5.76 (2.28-14.55) | 2 (5.0) | 3 (7.1) | 1.50 (0.20-11.54) |
| $P_{trend}$ | | | <0.0001 | | | 0.22 |
| Age‡ | | | | | | |
| <55 | 100 (13.1) | 6 (15.0) | 1.00 (Reference) | 13 (32.5) | 1 (2.3) | |
| 55-64 | 197 (25.8) | 14 (35.0) | 1.18 (0.44-3.18) | 6 (15.0) | 5 (11.6) | 1.00 (Reference) |
| 65-70 | 189 (24.8) | 10 (25.0) | 0.88 (0.31-2.50) | 5 (12.5) | 13 (30.2) | 8.23 (2.07-32.75) |
| 71-79 | 277 (36.3) | 10 (25.0) | 0.60 (0.21-1.70) | 16 (40.0) | 24 (55.8) | 4.75 (1.56-14.48) |
| $P_{trend}$ | | | 0.16 | | | 0.009 |
| Gender | | | | | | |
| Male | 419 (54.9) | 16 (40.0) | 1.00 (Reference) | 20 (50.0) | 18 (41.9) | 1.00 (Reference) |
| Female | 344 (45.1) | 24 (60.0) | 1.83 (0.96-3.49) | 20 (50.0) | 25 (58.1) | 1.39 (0.58-3.30) |
| Differentiation§ | | | | | | |
| Well | 71 (10.1) | 2 (5.3) | | 3 (8.1) | 1 (2.4) | |
| Moderate | 526 (74.6) | 23 (60.5) | 1.00 (Reference) | 20 (54.1) | 23 (54.8) | 1.00 (Reference) |
| Poor | 108 (15.3) | 13 (34.2) | 2.87 (1.43-5.79) | 14 (37.8) | 18 (42.9) | 1.23 (0.50-3.04) |
| Mucinous histology | | | | | | |
| No | 685 (89.8) | 30 (75.0) | 1.00 (Reference) | 29 (72.5) | 28 (66.7) | 1.00 (Reference) |
| Yes | 78 (10.2) | 10 (25.0) | 2.93 (1.38-6.22) | 11 (27.5) | 14 (33.3) | 1.32 (0.51-3.39) |
| Colorectal family history‖ | | | | | | |
| No | 284 (79.6) | 9 (47.4) | 1.00 (Reference) | 13 (59.1) | 17 (68.0) | 1.00 (Reference) |
| Yes | 73 (20.4) | 10 (52.6) | 4.23 (1.65-10.84) | 9 (40.9) | 8 (32.0) | 0.64 (0.18-2.19) |
| *Ki-ras* | | | | | | |
| WT | 458 (63.6) | 37 (97.4) | 1.00 (Reference) | 27 (75.0) | 43 (100.0) | |
| Mutant | 262 (36.4) | 1 (2.6) | 0.05 (<0.01-0.35) | 9 (25.0) | 0 (0.0) | |
| *p53* | | | | | | |
| WT | 369 (51.0) | 21 (53.8) | 1.00 (Reference) | 35 (89.7) | 36 (85.7) | 1.00 (Reference) |
| Mutant | 355 (49.0) | 18 (46.2) | 0.89 (0.47-1.70) | 4 (10.3) | 6 (14.3) | 1.46 (0.38-5.61) |
| CIMP | | | | | | |
| Low | 554 (78.7) | 3 (8.6) | 1.00 (Reference) | 11 (32.4) | 2 (4.7) | 1.00 (Reference) |
| High | 150 (21.3) | 32 (91.4) | 39.39 (11.90-130.41) | 23 (67.6) | 41 (95.3) | 9.80 (2.00-48.10) |

*OR for *BRAF* mutant status associated with this characteristic, from univariate analysis except as noted.
†Stages 1 and 2 were combined in the calculation of ORs due to small cell size.
‡The youngest two age groups were combined to allow the calculation of ORs.
§Well and moderate differentiation were combined in the calculation of the ORs due to small cell size.
‖Age adjusted.

DNA extracted from tumors microdissected for previous studies (4). Methylation-specific PCR was then done as described previously for the following CpG islands: MINT 1, MINT 2, MINT 31, *p16*, and *hMLH1* (12). This panel was being used at the time our study began by the group that originally described CIMP and its importance in colorectal cancer, and their criterion for CIMP high was methylation of two or more of these CpG islands (12, 13). CIMP low was defined as less than two of five markers methylated. Sufficient DNA for CIMP determination was present for 838 tumors with *BRAF* V600E results and 26 tumors without *BRAF* V600E results.

***Ki-ras*, *p53*, and microsatellite instability.** Codon 12 and 13 *Ki-ras* mutations, *p53* mutations in exons 5 to 8, and MSI were determined in previous studies (4, 14, 15). These studies preceded the development of the Bethesda consensus panel; the MSI markers used were BAT-26 (a mononucleotide repeat, which by itself is a very good measure of generalized instability), *TGFβRII* (a coding mononucleotide repeat, which is unstable in most colorectal cancers with MSI), and a panel of 10 tetranucleotides repeats, which show a high correlation with the Bethesda consensus panel and BAT-26 (16). A hierarchical approach was then used for MSI determination; 824 tumors were classified (either stable or unstable) for BAT-26, 59 tumors (which did not show results for BAT-26) were classified using *TGFβRII*, and 3 tumors (which showed no results for either BAT-26 or *TGFβRII*) were classified using the panel of 10 tetranucleotide repeats; in that case, if ≥30% of the 10 tetranucleotide repeats were unstable, the tumor was classified as unstable, and if <30% were unstable, the tumor was classified as stable. MSI could not be determined for 25 tumors.

**Statistical analysis.** Univariate relationships between *BRAF* V600E mutation and age at diagnosis, tumor site, AJCC stage, gender, grade of differentiation, a histologic classification of mucinous, mucin-producing, or signet ring, family history of colorectal cancer (defined as colorectal cancer in a first-degree relative), mutations in *Ki-ras* and *p53*, and CIMP were evaluated using logistic regression to calculate odds ratios (OR). All ORs were unadjusted, except for family history of colorectal cancer, which was adjusted for age at diagnosis. Survival data were available for 930 individuals with CIMP and/or *BRAF* results; 905 of these had *BRAF* mutational data and 857 of these had CIMP data. Five-year survival was evaluated using Kaplan-Meier plots for mortality due to all causes. Associations and interactions between *BRAF*, CIMP, and survival were evaluated among microsatellite-stable tumors using Cox proportional hazards models adjusting for age at diagnosis, AJCC stage, and tumor site. Median follow-up time was 65 months. All data analyses were done using SAS version 8.2 (SAS Institute, Cary, NC).

## Results

***BRAF* and microsatellite-stable cancers.** The V600E *BRAF* mutation was detected in 9.5% (87 of 911) of colon cancers overall and 5% (40 of 803) of microsatellite-stable cancers. Relationships between *BRAF* V600E mutation and clinicopathologic variables and tumor mutations in microsatellite-stable tumors are shown in Table 1. Significant relationships were seen between the V600E *BRAF* mutation and CIMP high, family history of colorectal cancer [OR, 4.23; 95% confidence interval (95% CI), 1.65-10.84], *Ki-ras* wild-type (WT), higher AJCC stage, poor differentiation, mucinous histology, and proximal tumor location. A nonsignificant excess of *BRAF* mutations was observed for females. The relationship to CIMP high was particularly strong (OR, 39.39; 95% CI, 11.90-130.41), as 91.4% of *BRAF* mutated tumors were CIMP high compared with 21.3% of *BRAF* WT. *BRAF* and *Ki-ras* mutations were, with one exception, mutually exclusive. *BRAF* mutations were not related to increased age.

***BRAF* and microsatellite-unstable cancers.** The *BRAF* V600E mutation was seen in 51.8% (43 of 83) of microsatellite-unstable cancers. The comparison of the V600E *BRAF* mutation to *BRAF* WT with respect to clinicopathologic variables and tumor mutations in microsatellite-unstable tumors is shown in Table 1. Some of this analysis was hampered by the relatively small number of unstable tumors, but unstable tumors with the V600E *BRAF*

**Table 2.** Univariate relationships with 5-year survival

| | 5-y overall survival (%) |
|---|---|
| Age at time of diagnosis | |
| <55 | 58.5 |
| 55-64 | 63.5 |
| 65-70 | 64.8 |
| 71-79 | 52.9 |
| *P* | 0.02* |
| Gender | |
| Male | 59.0 |
| Female | 59.7 |
| *P* | 0.98 |
| Tumor site | |
| Proximal | 55.6 |
| Distal | 63.7 |
| *P* | <0.01* |
| AJCC stage | |
| 1 | 83.7 |
| 2 | 75.3 |
| 3 | 55.4 |
| 4 | 8.7 |
| *P* | <0.01* |
| Differentiation | |
| Well | 78.6 |
| Moderate | 60.6 |
| Poor | 46.2 |
| *P* | <0.01* |
| MSI | |
| Stable | 57.7 |
| Unstable | 75.9 |
| *P* | <0.01* |
| CIMP | |
| Low | 60.2 |
| High | 55.5 |
| *P* | 0.12 |
| CIMP (stable) | |
| Low | 60.2 |
| High | 48.7 |
| *P* | <0.01* |
| CIMP (unstable) | |
| Low | 71.4 |
| High | 74.6 |
| *P* | 0.77 |
| *BRAF* | |
| WT | 60.7 |
| Mutant | 47.5 |
| *P* | <0.01* |
| *BRAF* (stable) | |
| WT | 60.0 |
| Mutant | 16.7 |
| *P* | <0.01* |
| *BRAF* (unstable) | |
| WT | 75.0 |
| Mutant | 76.2 |
| *P* | 0.89 |

NOTE: *P* is a log-rank statistic for differences in 5-year survival.
*Difference in survival rates are statistically significant.

**Table 3.** HRRs for risk of dying from all causes comparing microsatellite-stable tumors with and without the V600E *BRAF* mutation and with and without CIMP high

| | *BRAF* | | | CIMP | | |
|---|---|---|---|---|---|---|
| | WT ($n$ = 758) deaths/ person-years* | V600E mutant ($n$ = 40) deaths/ person-years | HRR (95% CI) | Low ($n$ = 570) deaths/ person-years | High ($n$ = 186) deaths/ person-years | HRR (95% CI) |
| Age adjusted | 299/2,788 | 33/70 | 3.97 (2.76-5.71) | 223/2,101 | 95/596 | 1.45 (1.14-1.84) |
| Age, AJCC stage, and tumor site adjusted | 299/2,788 | 33/70 | 2.97 (2.05-4.32) | 223/2,101 | 95/596 | 1.13 (0.87-1.47) |
| Age adjusted by AJCC stage | | | | | | |
| 1 | 32/854 | 0/5 | Undefined | 23/686 | 8/120 | 1.77 (0.79-4.00) |
| 2 | 47/889 | 4/17 | 4.88 (1.73-13.76) | 36/676 | 12/171 | 1.25 (0.65-2.40) |
| 3 | 91/802 | 15/38 | 3.60 (2.07-6.25) | 72/554 | 35/253 | 1.00 (0.67-1.51) |
| 4 | 126/203 | 14/10 | 2.04 (1.16-3.59) | 89/150 | 40/52 | 1.31 (0.89-1.92) |

*Person-years is the sum of each person's time at risk (time from diagnosis to death or censoring, maximum of 5 years), in years.

mutation clearly show the same relationship as microsatellite-stable mutant tumors with respect to CIMP high and lack (in this case, complete) of *Ki-ras* mutations. There is a suggestion of other relationships shown by stable tumors, such as proximal location, increased stage, and mucinous histology, but these were not statistically significant. One difference between unstable *BRAF* mutated tumors and stable mutated tumors is that unstable tumors with the V600E *BRAF* mutation were associated with increased age.

**Five-year survival.** Univariate relationships between several clinicopathologic variables and percent 5-year survival are shown in Table 2. Significant relationships with poor survival were seen with age, proximal tumor site, higher AJCC stage, and poor differentiation, whereas MSI was associated with an improved prognosis. Both the *BRAF* V600E mutation and CIMP high were associated with a poorer 5-year survival in microsatellite-stable tumors (16.7% and 48.7%, respectively). Among CIMP high microsatellite-stable tumors, the V600E *BRAF* mutation was associated with a significantly poorer 5-year survival (14.6% versus 55.8%; $P < 0.0001$). Microsatellite-unstable tumors with or without the V600E *BRAF* mutation were associated with an excellent percent 5-year survival (76.2 and 75.0%, respectively).

**Microsatellite-stable tumors, *BRAF* mutations, CpG island methylator phenotype, and survival.** Of the 820 individuals with microsatellite-stable cancers, 342 were no longer living 5 years after diagnosis. 256 died of colon cancer, 29 died of another type of cancer, 47 died of another cause, and the cause of death was unspecified for 10 individuals. The effects of the V600E *BRAF* mutation and CIMP on the likelihood of dying from all causes in microsatellite-stable tumors are shown in Table 3. *BRAF* mutated tumors were associated with a significantly higher risk of dying than *BRAF* WT tumors in an analysis adjusted for age [hazard rate ratio (HRR), 3.97; 95% CI, 2.76-5.71], in an analysis adjusted for age, stage, and tumor site (HRR, 2.97; 95% CI, 2.05-4.32), and in stage-specific, age-adjusted analyses for AJCC stages 2 to 4 (HRRs, 4.88, 3.60, and 2.04, respectively). CIMP high tumors were also associated with a significantly higher HRR in age-adjusted analyses, although the point estimate was lower than for *BRAF* (1.45 versus 3.97). Adjustment for age, stage, and tumor site eliminated the association of CIMP and survival (HRR, 1.13; 95% CI, 0.87-1.47),

**Table 4.** HRRs for risk of dying of colon cancer comparing microsatellite-stable tumors with and without the V600E *BRAF* mutation and with and without CIMP high

| | *BRAF* | | | CIMP | | |
|---|---|---|---|---|---|---|
| | WT ($n$ = 758) deaths/ person-years | V600E mutant ($n$ = 40) deaths/ person-years | HRR (95% CI) | Low ($n$ = 570) deaths/ person-years | High ($n$ = 186) deaths/ person-years | HRR (95% CI) |
| Age adjusted | 220/2,788 | 30/70 | 4.57 (3.11-6.73) | 160/2,101 | 78/596 | 1.70 (1.29-2.23) |
| Age, AJCC stage, and tumor site adjusted | 220/2,788 | 30/70 | 3.19 (2.14-4.75) | 160/2,101 | 78/596 | 1.30 (0.96-1.74) |
| Age adjusted by AJCC stage | | | | | | |
| 1 | 7/854 | 0/5 | Undefined | 5/686 | 2/120 | 1.74 (0.33-9.30) |
| 2 | 27/889 | 3/17 | 5.83 (1.74-19.53) | 20/676 | 9/171 | 1.76 (0.80-3.88) |
| 3 | 70/802 | 13/38 | 4.06 (2.23-7.39) | 52/554 | 30/253 | 1.23 (0.78-1.93) |
| 4 | 114/203 | 14/10 | 2.28 (1.29-4.02) | 81/150 | 37/52 | 1.37 (0.92-2.06) |

and no significant relationships between CIMP high and risk of dying were seen in AJCC stage-specific analyses.

The effects of the V600E *BRAF* mutation and CIMP on the likelihood of dying from colon cancer (as opposed to death from all causes) in microsatellite-stable tumors are shown in Table 4. The same relationships of the *BRAF* V600E mutation and increased likelihood of dying are seen as with overall survival in Table 3; indeed, the HRRs are even higher. CIMP high shows similarly minimal (and mostly nonsignificant) associations with increased risk of dying from colon cancer as were seen with death from all causes. Adjustment for family history of colorectal cancer did not significantly alter these results or those above for death from all causes (data not shown).

Multivariate analyses of the effect of *BRAF* mutations adjusted for CIMP high and CIMP high adjusted for *BRAF* mutations show that the deleterious effects on survival in microsatellite-stable cancers (either overall or colorectal) are entirely attributable to *BRAF* mutations (Table 5). This is further exemplified by the analysis of the four permutations of *BRAF* and CIMP (*BRAF* WT, CIMP low; *BRAF* mutant, CIMP low; *BRAF* WT, CIMP high; and *BRAF* mutant, CIMP high) and their effect on survival (Table 5); again, it is mutant *BRAF* rather than CIMP high that increases the risk of death.

Kaplan-Meier survival estimates comparing the overall survival of V600E *BRAF* mutation versus *BRAF* WT for microsatellite-stable colon cancers are shown in Fig. 1. Although relatively few numbers of tumors are present at each stage, tumors with the V600E *BRAF* mutations had a significantly higher risk of death than *BRAF* WT tumors for stages 2 to 4 ($P < 0.01$, log-rank test).

The Kaplan-Meier survival estimates comparing the overall survival of CIMP high and CIMP low for microsatellite-stable tumors is shown in Fig. 2. Significant relationships were not seen for any AJCC stage ($P = 0.09$, 0.40, 0.73, and 0.14 for stages 1-4, respectively).

**Microsatellite-unstable cancers.** Of the 83 individuals with microsatellite-unstable colon cancers, 8 died of colon cancer, 3 died of another type of cancer, 6 died of another cause, and the cause of death was unspecified for 3 individuals. HRRs and Kaplan-Meier survival estimates for microsatellite-unstable tumors with and without the *BRAF* V600E mutation were not calculated because so few colon cancer deaths (3 and 5, respectively) occurred in these groups.

**Other *BRAF* mutations.** Four other *BRAF* mutations were identified (Table 6) but were not used in the above analyses. Three of the mutations occurred in codon 594 (D594N, D594G, and D594G) and were present in microsatellite-stable, CIMP low tumors. All of these individuals survived at least 5 years (although all were relatively low-stage tumors). The fourth mutation (G606R) occurred in a microsatellite-stable, CIMP high tumor and was associated with death due to colon cancer after 19 months.

## Discussion

Over 90% of colon cancers with the *BRAF* V600E mutation were CIMP high (Table 1). In microsatellite-stable cancers, tumors with the V600E mutation also showed many of the same clinicopathologic relationships observed previously in CIMP high colon cancers in general, including proximal tumor location, increased AJCC stage, poor differentiation, and mucinous histology (Table 1; refs. 17, 18).[4] The major difference was that the V600E *BRAF* mutation was not related to increased age in stable tumors; several previous studies have noted a relationship between CIMP high and older age in stable tumors (17, 18).[4] The relationship between CIMP high and older age remains in our data set if *BRAF* mutated tumors are excluded (data not shown). Another potential difference is the impressively increased risk of a positive family history of colorectal cancer associated with the *BRAF* V600E mutation in microsatellite-stable cancers (OR, 4.23; 95% CI, 1.65-10.84). There has been a recent study that related *BRAF* mutations to colorectal cancer-prone families whose tumors exhibited varying degrees of MSI (19), so there is precedence for a relationship between *BRAF* mutations and family history of colon cancer (although the relationship we observed was only with microsatellite-stable tumors). In two previous studies of CIMP and family history of all types of cancer,

**Table 5.** Joint analysis of *BRAF* and CIMP status and survival of individuals with microsatellite-stable colon cancers

| | Colorectal cancer survival | | | Overall survival | |
|---|---|---|---|---|---|
| | *n* | Deaths/ person-years | HRR* (95% CI) | Deaths/ person-years | HRR* (95% CI) |
| *BRAF*† | | | | | |
| WT | 758 | 220/2,788 | 1.00 (Reference) | 299/2,788 | 1.00 (Reference) |
| Mutant | 40 | 30/70 | 3.11 (2.04-4.74) | 33/70 | 3.06 (2.06-4.54) |
| CIMP‡ | | | | | |
| Low | 570 | 160/2,101 | 1.00 (Reference) | 223/2,101 | 1.00 (Reference) |
| High | 186 | 78/596 | 0.97 (0.70-1.36) | 95/596 | 0.88 (0.66-1.18) |
| *BRAF* WT/CIMP low | 549 | 153/2,037 | 1.00 (Reference) | 213/2,037 | 1.00 (Reference) |
| *BRAF* mutant/CIMP low | 3 | 2/6 | 20.78 (4.85-89.11) | 2/6 | 11.14 (2.66-46.71) |
| *BRAF* WT/CIMP high | 150 | 53/530 | 1.01 (0.72-1.42) | 66/530 | 0.91 (0.68-1.22) |
| *BRAF* mutant/CIMP high | 32 | 24/52 | 3.60 (2.29-5.66) | 27/52 | 3.17 (2.08-4.83) |

*Adjusted for age, AJCC stage, and tumor site.
†HRR also adjusted for CIMP.
‡HRR also adjusted for *BRAF*.

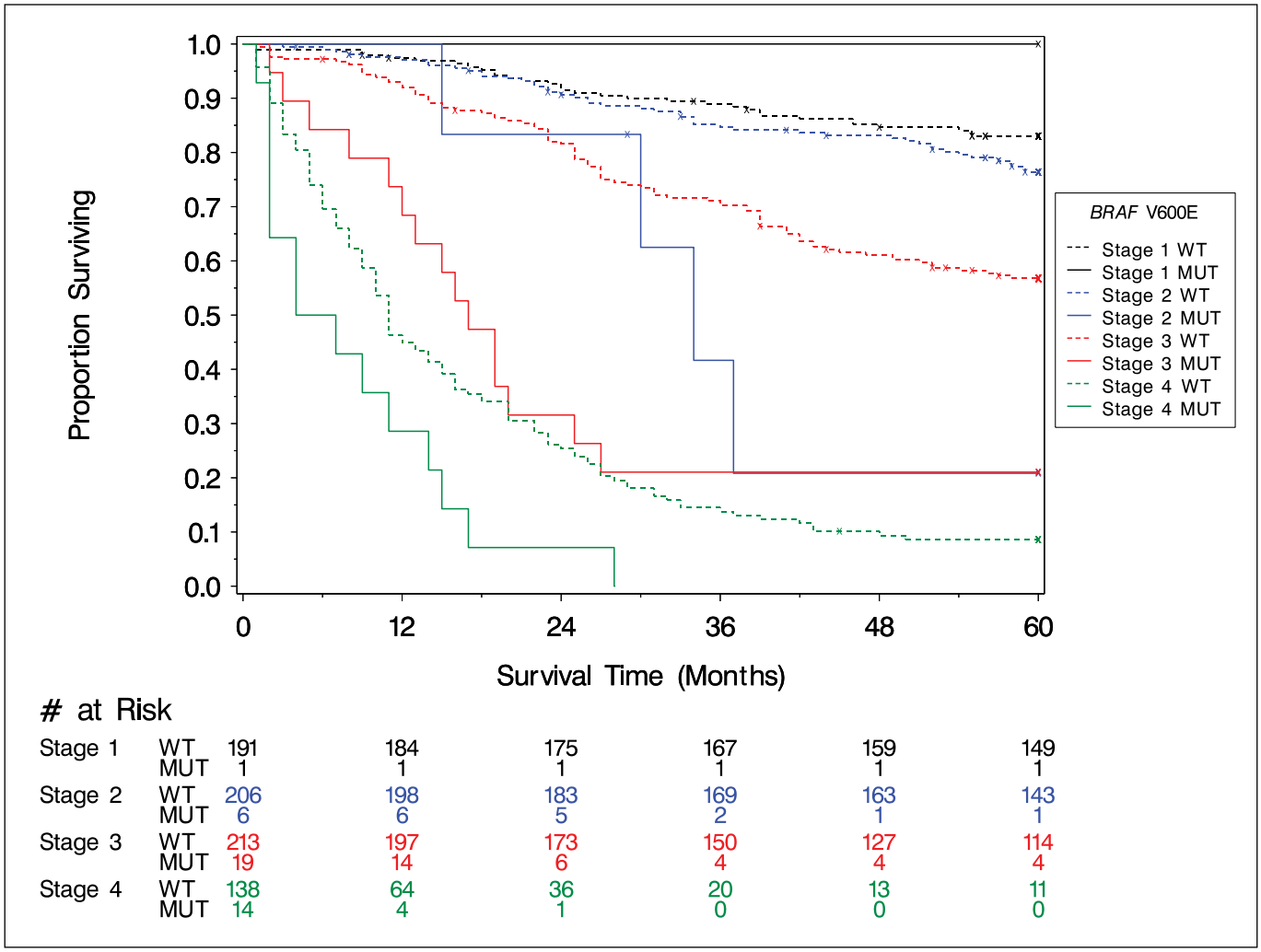


**Figure 1.** Kaplan-Meier survival estimates of individuals with microsatellite-stable colon cancers by stage and V600E *BRAF* mutation status. *X*, individuals who were lost to follow-up.

one showed a significant relationship (13) and the other showed a nonsignificant trend, which apparently disappeared in a multivariate analysis (20); we previously reported a small but nonsignificant relationship between CIMP high and family history of colorectal cancer and no relationship between CIMP and all types of cancer.[4] Finally, another difference is that stable tumors with the V600E *BRAF* mutation did not show the increased frequency of *Ki-ras* mutations that we and others have reported with CIMP in stable tumors; this is not surprising given that these mutations are, for the most part, mutually exclusive (Table 1; refs. 2, 8).

The evaluation of the V600E *BRAF* mutation in unstable tumors was hampered by the relatively small numbers of such cancers. Still, and in contrast with stable tumors, the V600E *BRAF* mutation did show the relationship to increased age typically exhibited by CIMP high colon cancer (18).[4]

The V600E *BRAF* mutation was present in 5% (40 of 803) of microsatellite-stable colon cancers and was associated with a significantly worse survival than microsatellite-stable tumors without this mutation. This was true in a univariate analysis after adjustment for age, stage, and tumor site, in stage-stratified analyses for AJCC stages 2 to 4, and in a Kaplan-Meier analysis for stages 2 to 4 (Tables 2, 3, and 4; Fig. 1). Because >90% of microsatellite-stable tumors with the V600E *BRAF* mutation are also CIMP high, it is important to see whether this poorer survival was specifically related to this mutation or whether it was simply a function of CIMP. Although CIMP high stable tumors were associated with a significantly worse 5-year survival than CIMP low stable tumors in univariate and age-adjusted analyses, the effect of CIMP high on survival was less than that seen with *BRAF* (Tables 2 and 3). In addition, and in contrast to the *BRAF* V600E mutation, no significant relationships were seen for CIMP in either an analysis adjusted for age, stage, and tumor site, in stage-stratified analyses, or in a Kaplan Meier analysis (Table 3; Fig. 2). The relatively minor effects of CIMP high on survival we observed are consistent with a previous study (18) and suggest that the effect of the V600E mutation on survival in stable tumors is not dependent on CIMP. In a direct comparison of CIMP high stable tumors with and without the V600E mutation, tumors with the V600E mutation had a significantly worse 5-year survival (14.6% versus 55.8%; $P < 0.0001$), and in multivariate analyses of CIMP and *BRAF*, only *BRAF* mutations had an effect on survival (Table 5).

The V600E mutation did not have the same effect on survival in tumors with MSI, as unstable tumors with and without this

mutation were associated with an excellent 5-year survival (76.2% and 75.0%, respectively). This emphasizes that it is not the V600E mutation per se that confers a poor prognosis but rather that the mutation has different effects depending on the genetic background in which it occurs and/or, perhaps, the oncogenic pathway that led to the development of the cancer. It should be stressed that this difference is not due to the presence or absence of CIMP, as >90% of both microsatellite-unstable and microsatellite-stable tumors with the V600E *BRAF* mutation are CIMP high (Table 1).

There has recently been a great deal of speculation that microsatellite-unstable colorectal cancers may develop from a subset of hyperplastic polyps (which often have *BRAF* mutations and are CIMP high) rather than from traditional adenomas (8). If this is true, then perhaps microsatellite-stable tumors with *BRAF* mutations develop from a different pathway (e.g., adenomas with *BRAF* mutations; ref. 2) or that both develop from the same serrated, hyperplastic polyp pathway but diverge with respect to clinical aggressiveness with the methylation of *hMLH1* in a subset of tumors that then develop MSI. Future studies of relevant precursor lesions will be necessary to address this question.

Four other *BRAF* mutations were identified (Table 5). Three of them encode substitutions of uncharged for charged amino acids at codon 594. A similar mutation (D594V) has been shown to lead to impaired kinase activity of *BRAF* and loss of the ability to activate extracellular signal-regulated kinase (21). Interestingly, all three of these mutations occurred in CIMP low tumors and all three individuals survived at least 5 years (although all were relatively low stage tumors). The fourth mutation (G606R) was not analyzed by Wan et al., but it is similar to other activating mutations described in that study (including the V600E mutation) in that it occurred in the activation segment of the protein and was a substitution of a charged for an uncharged amino acid. This mutation occurred in a microsatellite-stable, CIMP high tumor and the individual died of colon cancer after 19 months, similar to the CIMP high context, increased stage, and poor survival we have seen with the more common V600E mutation.

Our findings with respect to the deleterious effect of *BRAF* mutations in microsatellite-stable cancers could have important clinical implications, especially if validated by other studies. Individuals with such tumors may be treated more aggressively, especially those with stage II cancers who typically do not receive chemotherapy at the present time. It may also be possible to

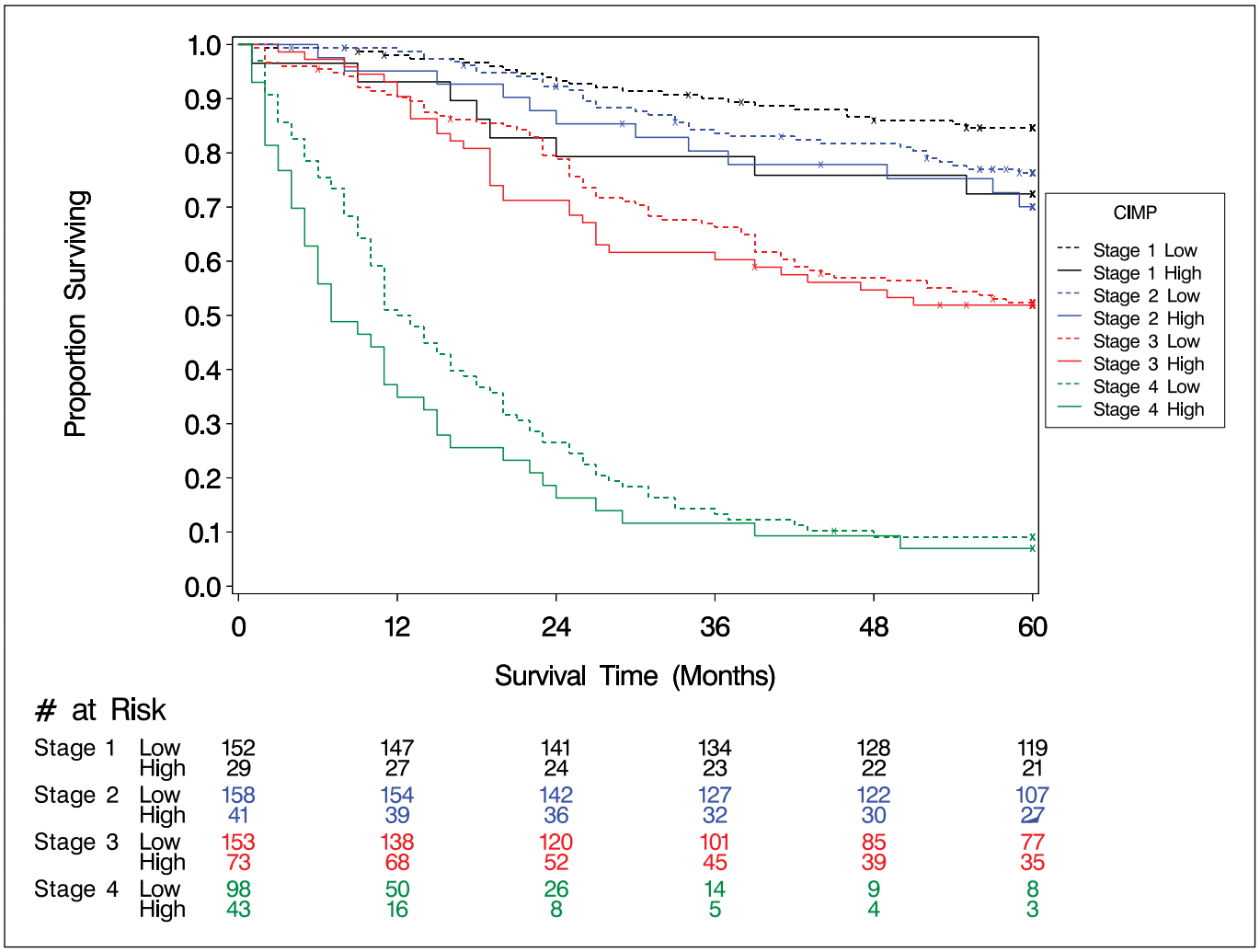


**Figure 2.** Kaplan-Meier survival estimates of individuals with microsatellite-stable colon cancers by stage and CIMP status. *X*, individuals who were lost to follow-up.

**Table 6.** Other *BRAF* mutations

| Mutation | Months of follow-up | Cause of death | MSI | CIMP | AJCC stage |
|---|---|---|---|---|---|
| D594N | 67 | Other cancer | Stable | Low | 2 |
| G606R | 19 | Colon cancer | Stable | High | 3 |
| D594G | 100 | Alive | Stable | Low | 1 |
| D594G | 94 | Alive | Stable | Low | 1 |

develop specific therapies for these tumors. It is possible that these therapies would not involve any that are specifically directed against mutated *BRAF*, as these mutations do not have an effect on survival in microsatellite-unstable cancers. It is also possible, however, that in the right genetic background therapy directed against this oncogenic mutation could have beneficial effects.

In summary, this large population-based study showed that microsatellite-stable colon cancers with the V600E *BRAF* mutation differed from other CIMP high stable tumors by showing no relationship with increased age and by showing a strong relationship to a family history of colorectal cancer. This latter finding suggests that future exploration of the genetic and/or environmental factors which relate to this association, may be fruitful. In addition, microsatellite-stable tumors with the V600E *BRAF* mutation had a significantly worse overall survival than stable tumors without this mutation and specifically showed poorer survival in AJCC stages 2 to 4. This poor survival was not related to the CIMP high phenotype, and the V600E mutation did not affect the excellent prognosis of microsatellite-unstable carcinomas. Further research is needed to determine the mechanism through which this mutation is associated with poor survival in microsatellite-stable colon cancers and/or whether there is a novel carcinogenic pathway associated with these genetic changes in microsatellite-stable tumors which is particularly aggressive.

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