Published in final edited form as:

Am J Surg Pathol. 2012 May ; 36(5): 744–752. doi:10.1097/PAS.0b013e31824430d7.

BRAF-mutated, Microsatellite-stable Adenocarcinoma of the Proximal Colon: An Aggressive Adenocarcinoma With Poor Survival, Mucinous Differentiation, and Adverse Morphologic Features

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Abstract

The association of *BRAF*V600E mutation and the presence of the CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) often confound analysis of *BRAF* mutation status and survival in colorectal carcinoma. We evaluated a consecutive series of proximal colonic adenocarcinomas for mismatch repair protein abnormalities/MSI, *BRAF*V600E mutation, and *KRAS* mutations in an attempt to determine the prognostic significance of these abnormalities and to correlate histopathologic features with molecular alterations. Of the 259 proximal colon adenocarcinomas analyzed for mismatch repair protein abnormalities and/or MSI, 181 proximal colonic adenocarcinomas demonstrated proficient DNA mismatch repair using either MSI PCR (n = 78), mismatch repair protein immunohistochemistry (n = 91), or both MSI PCR and mismatch repair immunohistochemistry (n = 12); these were tested for the *BRAF*V600E mutation and *KRAS* mutations. Compared with *BRAF* wild-type adenocarcinomas, *BRAF*-mutated adenocarcinomas more frequently demonstrated adverse histologic features such as lymphatic invasion (16/20, 80% vs. 75/161, 47%; $P = 0.008$), mean number of lymph node metastases (4.5 vs. 2.2; $P = 0.01$), perineural invasion (8/20, 40% vs. 13/161, 8%; $P = 0.0004$), and high tumor budding (16/20, 80% vs. 83/161, 52%; $P = 0.02$). *BRAF*-mutated adenocarcinomas frequently contained areas with mucinous histology ($P = 0.0002$) and signet ring histology ($P = 0.03$), compared with *KRAS*-mutated and *KRAS/BRAF* wild-type adenocarcinomas. Clinical follow-up data were available for 173 proximal colonic adenocarcinomas with proficient DNA mismatch repair. Patients with *BRAF*-mutated adenocarcinomas had a median survival of 12.3 months with a 1-year probability of survival of 54% and a 1-year disease-free survival of 56%. Patients with *KRAS*-mutated and *KRAS/BRAF* wild-type adenocarcinomas had significantly improved overall

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Conflicts of Interest and Source of Funding: The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

survival (unadjusted log-rank $P=0.03$ and unadjusted log-rank $P=0.0002$, respectively) and disease-free survival (unadjusted log-rank $P=0.02$ and unadjusted log-rank $P=0.02$, respectively) compared with patients with *BRAF*-mutated adenocarcinomas. When adjusting for tumor stage, survival analysis demonstrated that patients with *BRAF*-mutated adenocarcinoma had a significantly poor overall survival and disease-free survival (hazard ratios 6.63, 95% CI, 2.60–16.94; and 6.08, 95% CI, 2.11–17.56, respectively) compared with patients with *KRAS/BRAF* wild-type adenocarcinomas. No significant difference in overall or disease-free survival was identified between patients with *KRAS*-mutated and *KRAS/BRAF* wild-type adenocarcinomas. Our results demonstrate that *BRAF*-mutated proximal colon adenocarcinomas with proficient DNA mismatch repair have a dismal prognosis with an aggressive clinical course and often display mucinous differentiation, focal signet ring histology, and other adverse histologic features such as lymphatic and perineural invasion and high tumor budding.

Keywords

BRAF; *KRAS*; MSI; mismatch repair; colon; adenocarcinoma; proximal

The predictive power of mutation analysis of the *Ras/Raf/MAPK* signaling pathway in colorectal carcinoma and response to antiepidermal growth factor receptor (EGFR) therapy have been well established.^{1,10,16,22} Mutations in *KRAS* are observed in 30% to 40% of colorectal carcinomas and lead to a constitutively activated kinase resulting in EGFR-independent inactivation of the *MAPK* pathway.^{5,12,47} Indeed, most studies have shown that activating *KRAS* mutations predict a lack of response to the anti-EGFR monoclonal antibodies cetuximab and panitumumab.^{1,16,22} *BRAF* is another component of the *Ras/Raf/MAPK* signaling pathway that is also frequently mutated in colorectal carcinoma but has a lower prevalence in colorectal carcinoma, seen in approximately 15% to 17% of colorectal carcinomas.^{30,46} *BRAF*V600E mutations are frequently observed in colorectal carcinomas demonstrating high levels of promoter methylation of CpG islands (CIMP-high, CIMP-H), with approximately 60% to 80% of CIMP-H tumors harboring mutations in *BRAF*.^{3,26,30} These *BRAF*-mutated CIMP-H colorectal carcinomas are also often characterized by high levels of microsatellite instability (MSI-H) due to the presence of a CpG island in the promoter region of the mismatch repair gene *MLH1*.^{8,48} Recent evidence suggests that activating *BRAF*V600E mutations also predict lack of response to anti-EGFR therapy.¹⁰

The prognostic importance of mutations in the EGFR signaling pathway is not entirely clear, with conflicting data reported in the literature. In some studies, the presence of *KRAS* mutations is associated with shorter survival.^{2,4,35} However, in these series demonstrating survival differences in *KRAS* mutated colorectal carcinoma, there are conflicting data on whether codon 12^{2,4} or codon 13³⁵ *KRAS* mutations confer prognostic significance. Most literature data indicate no survival difference between *KRAS* wild-type and *KRAS*-mutated colorectal carcinomas.^{29,34} Assessing the importance of *BRAF* mutation status and prognosis is made much more difficult given the close association of *BRAF*V600E mutations and the presence of CIMP-H and sporadic MSI-H.^{8,48} MSI-H status is generally associated with improved overall and disease-free survival,³¹ although not all studies have verified the association of MSI-H and improved overall survival.²³ Thus, the presence of

MSI-H often confounds analysis of *BRAF* mutation status and survival in colorectal carcinoma. The studies analyzing both MSI and *BRAF* mutation status have shown that *BRAF*V600E mutation is associated with poor survival in microsatellite-stable tumors but has no effect on the improved survival of MSI-H colorectal adenocarcinomas.^{20,30,36} In contrast, the reports in the literature that did not analyze the prognostic significance of *BRAF* status independent of MSI status failed to identify an association between *BRAF* V600E mutation and reduced survival.^{17,20,33} In addition, most studies lack a rigorous analysis of the histopathologic features of the colorectal carcinomas included, and correlation of the histopathologic findings with mutation status in the EGFR signaling pathway is often not performed.

We analyzed proximal colon adenocarcinomas for mismatch repair protein abnormalities/MSI, *BRAF* V600E mutation, and *KRAS* mutations in an attempt (1) to determine the prognostic significance of mutations in the EGFR signaling pathway and (2) to correlate histopathologic features with molecular alterations. We limited our analysis to proximal colonic adenocarcinomas, as the molecular pathway to colorectal carcinoma appears distinct in the proximal and distal colon.^{30,36} Our results indicate that proximal colon adenocarcinomas with *BRAF* mutations have distinct morphologic characteristics and that *BRAF*-mutated proximal colonic adenocarcinomas with proficient DNA mismatch repair are associated with an aggressive clinical course.

MATERIALS AND METHODS

Study Group

The clinicopathologic records of 181 patients with primary invasive adenocarcinoma of the proximal colon, including the cecum, ascending colon, and transverse colon, accessioned at the Departments of Pathology at Stanford University Hospital for the years 2005 to 2010 and at Cleveland Clinic from 2009 to 2010, were reviewed. Specifically excluded from this study were neuroendocrine tumors and adenocarcinomas of the appendix. Further, 8 subjects did not have adequate information to determine vital status and/or date of last contact. Therefore, the survival analysis was limited to 173 proximal colonic adenocarcinomas. Pathology reports and hospital charts were reviewed, and the following information was obtained: type of initial surgical procedure and the anatomic extent of tumor spread at initial presentation. Intraoperative and clinical follow-up data were obtained from hospital and clinic charts under the guidelines of the Stanford University and Cleveland Clinic Institutional Review Boards.

Pathologic Evaluation

A total of 727 (341 Stanford cases, 397 Cleveland Clinic cases) primary colorectal adenocarcinomas were surgically resected for the years 2005 to 2010 at Stanford University and from 2009 to 2010 at Cleveland Clinic. Of these, 259 were primary to the proximal colon, including 137 cases seen at Stanford University and 122 cases seen at Cleveland Clinic Hospital. All available cases of adenocarcinoma of the proximal colon with proficient DNA mismatch repair were histologically reviewed (n = 181), and the following histologic features were recorded for each tumor: grade, extent of invasion, lymph node metastases,

status of margin of resection, lymphatic invasion, perineural invasion, venous invasion, tumor budding, tumor-infiltrating lymphocytes, Crohn-like peritumoral reaction, proportion of tumor with mucinous histology, proportion of tumor with signet ring histology, presence and type of precursor lesion, and synchronous colorectal polyps. The grade of each colonic adenocarcinoma was scored using a 2-tiered scheme: low grade, with >50% gland formation, and high grade, with <50% gland formation. Tumor budding assessment was made using the rapid bud count method⁴⁹: high budding, in which >50% of areas examined under 200x magnification were positive for budding, and low budding, in which <50% of areas examined under 200x magnification were positive for budding. The presence of tumor-infiltrating lymphocytes was defined as >7 lymphocytes per 10 high-power fields.³⁸ Crohn-like peritumoral reaction was scored as absent, mild, or intense, as previously described.¹³ The presence or absence of precursor polyps and the histologic subtype of the precursor polyp associated with invasive colonic adenocarcinoma were recorded.^{15,39}

Mismatch Repair Protein Immunohistochemical Analysis and MSI Analysis Polymerase Chain Reaction

Mismatch repair protein immunohistochemistry was performed on a subset (n = 341, Stanford cases) of study cases using the standard streptavidin biotin peroxidase procedure. Primary monoclonal antibodies against MLH1 (clone G168-728, BD PharMingen, San Diego, CA, 1:200), MSH2 (clone FE11, Oncogene Research Products, Cambridge, MA, 1:100), MSH6 (clone 44, BD Transduction, San Jose, CA, 1:200), and PMS2 (clone MRQ-28, Cell Marque, Rocklin, CA, 1:10) were applied to 4-mm-thick formalin-fixed, paraffin-embedded sections. The sections were deparaffinized in xylene and rehydrated through graded alcohols to distilled water before undergoing antigen retrieval by heat treatment in either citrate solution pH 6.0 (MLH1, PMS2, and MSH2) or EDTA solution pH 9.0 (MSH6). An automated detection using a Leica Bond Autostainer (Buffalo Grove, IL) was carried out. Normal expression was defined as nuclear staining within tumor cells, using infiltrating lymphocytes as positive internal control. Negative protein expression was defined as complete absence of nuclear staining within tumor cells in the face of concurrent positive labeling in internal non-neoplastic tissues.

All Cleveland Clinic (386) and a subset of Stanford colonic adenocarcinomas were analyzed using the ProMega MSI analysis system using a panel of 5 mononucleotide microsatellite markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and 2 pentanucleotide repeats (Penta C and Penta D) incorporated into a multiplex fluorescence assay.⁴³ Briefly, the tests were conducted on tumor and normal DNA extracted from paraffin-embedded tissue blocks using either the DNease Tissue Kit (Qiagen, Valencia, CA) or the DNAIQ Case-work Pro Kit (Promega, Madison, WI); manual microdissection was performed, when required, to exclude overabundance of nonlesional tissue. Paired DNA samples from neoplastic and non-neoplastic samples were genotyped and analyzed by capillary electrophoresis using either an ABI 310 or ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). In accordance with NCI guidelines, MSI at 2 loci or more was defined as MSI-high (MSI-H), instability at a single locus was defined as MSI-low (MSI-L), and no instability at any of the loci tested was defined as microsatellite stable (MSS).

BRAF and KRAS Mutation Analysis

DNA was extracted from paraffin sections, using either the DNease Blood and Tissue Kit (Qiagen) (Stanford cases, n = 103) or the DNAIQ Casework Pro Kit (Promega) (Cleveland Clinic cases, n = 72) after xylene deparaffinization and ethanol wash. For all cases, *BRAF* mutation analysis at codon 600 (V600E) was performed by a real-time PCR based on an allelic discrimination method described previously.^{18,50} Briefly, real-time polymerase chain reaction (PCR) was performed using allele-specific primers designed to selectively amplify the wild-type (T1796) and mutant (A1796) *BRAF* alleles. The primer sequences were as follows: V, 5'-GTGATTTTGGTCTAGCTACtGT; E, 5'-CGCGGCCGCGCGCGCGGTGATTTTGGTCTAGCTACcGA; and AS, 5'-TAGCCTCAATTCTTACCATCCAC. PCR amplification and melting curve analysis were performed on an iCycler iQ (Biorad, Hercules, CA) or Applied Biosystems 7500. Genomic DNA was amplified in a 25µmL volume containing 1X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), forward primer V (300 nM), forward primer E (900 nM), and reverse primer AS (300 nM). The cycling conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. After amplification, samples were subjected to a temperature ramp from 60°C to 99°C, increasing by 1°C at each step. For wild-type samples, single peaks were observed at 80°C, whereas samples containing mutant alleles produced single peaks at 85°C.

For *KRAS* mutational analysis, 3 methods were utilized. For 103 Stanford cases, mutant *KRAS* was detected using a validated *KRAS* mutation kit (Entrogen, Tarzana, CA) that identifies the common somatic mutations located in codons 12, 13, and 61 of the *KRAS* gene. The mutations were detected on an Applied Biosystems 7900 (Applied Biosystems). Evaluation of *KRAS* assay results was performed according to the manufacturer's instructions. For 36 Cleveland Clinic cases, identification of point mutations in *KRAS* codons 12 and 13 was carried out using a Light Cycler 2.0 as described by Nikiforova et al.²⁷ In brief, a PCR amplicon was generated using primers (K-rasF1: 5'-AAGGCCTGCTGAAAATGACTG; K-rasR1: 5'-GGTCCTGCACCAGTAATATGCA) that flank the *KRAS* codon 12/13 mutational hotspot. Using a pair of fluorescently labeled anchor and sensor oligonucleotides (KRASanc1: 5'-CGTCCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGC-AGT-fluorescein; KRASsnsr1: 5'-LC Red640-TGCCTACGCCACCAGCTCCAA-phosphate) that hybridize adjacent to each other, amplification and mutation detection was accomplished through fluorescence resonance energy transfer. The sensor oligonucleotide spans the mutation site, allowing for the detection of a codon 12 or codon 13 mutation based on the distinct melting temperatures (T_m) of wild-type *KRAS* and mutant *KRAS*. For the other 36 Cleveland Clinic cases, *KRAS* mutational analysis was performed by PCR amplification of a 263 base-pair product that included codons 12 and 13, followed by both forward and reverse cycle sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). The following primers (Invitrogen/Gibco BRL) were used: *KRAS* Exon 1 forward primer = 5'-GGTGAGTTTGTATTAAAGGTACTGG and reverse primer = 5'-TCCTGCACCAGTAATATGCA.

Statistical Analysis

The primary endpoints selected for analysis were disease-free survival and overall survival. Disease-free survival was defined as the time (measured in days) from date of initial diagnosis to the date of first evidence of tumor recurrence. Overall survival was defined as the time (measured in days) from the date of initial diagnosis to the date of death or last follow-up. Survival analyses were performed using Kaplan-Meier methods with log-rank tests and with Cox regression methods. Univariate relationships between mutation status and continuous measures were assessed with 1-way ANOVA models, and associations between mutation status and categorical measures were assessed with the Fisher exact test or the Freedman-Halton extension of the Fisher exact test. All statistics were assessed using 2-sided tests with P-values <0.05 considered statistically significant. To help control for multiple comparisons, Bonferroni-adjusted P-values are also reported for the differences between mutation status in the log-rank test (KRAS vs. BRAF, BRAF vs. BRAF/KRAS wild type, and KRAS vs. BRAF/KRAS wild type). All statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

RESULTS

Clinicopathologic Features of Proximal Colon Adenocarcinomas Stratified by KRAS and BRAF Mutation Status

Of the 259 cases of proximal colonic adenocarcinoma analyzed for DNA mismatch repair abnormalities, 181 cases (70%), which constitute our study cases, were identified to be proficient in DNA mismatch repair using either MSI PCR (n = 78), mismatch repair protein immunohistochemistry (n = 91), or both MSI PCR and mismatch repair immunohistochemistry (n = 12). The clinicopathologic features of the 181 study cases stratified by *KRAS* and *BRAF* mutation status are detailed in Table 1. For all cases, the mean age was 67 years (range, 33 to 98 y) with a similar age distribution between groups stratified by *KRAS* and *BRAF* mutation status ($P = 0.20$). *BRAF*-mutated adenocarcinomas were seen more frequently in women (13/20, 65%) compared with *KRAS*-mutated adenocarcinomas (34/78, 44%) and *KRAS/BRAF* wild-type adenocarcinomas (39/83, 47%), although this did not reach statistical significance ($P = 0.23$). *BRAF*-mutated adenocarcinomas were more frequently stage III or IV (15/20, 75%) compared with *KRAS*-mutated adenocarcinomas (45/78, 57%) and *KRAS/BRAF* wild-type adenocarcinomas (43/83, 52%); however, the difference in stage between these groups was not statistically significant ($P = 0.45$). As seen in Table 1 and Figure 1, there are clear differences among the rates of adverse histologic features between *BRAF*-mutated, *KRAS*-mutated, and *KRAS/BRAF* wild-type adenocarcinomas, and differences among the three groups were driven by *BRAF*-mutated colonic adenocarcinoma. Compared with *BRAF* wild-type adenocarcinomas, *BRAF*-mutated adenocarcinomas more frequently demonstrated adverse histologic features such as lymphatic invasion (16/20, 80% vs. 75/161, 47%; $P = 0.008$), mean number of lymph node metastases (4.5 vs. 2.2; $P = 0.01$), perineural invasion (8/20, 40% vs. 13/161, 8%; $P = 0.0004$), and high tumor budding (16/20, 80% vs. 83/161, 52%; $P = 0.02$). *BRAF*-mutated adenocarcinomas also demonstrated a distinct morphologic appearance compared with *KRAS*-mutated and *KRAS/BRAF* wild-type adenocarcinomas (Fig. 1). *BRAF*-mutated adenocarcinomas frequently contained areas with abundant

extracellular mucin, which was either focal (<50% of tumor volume, 9 cases) or prominent (>50% of tumor volume, 6 cases). In addition, *BRAF*-mutated adenocarcinomas frequently demonstrated signet ring histology (4/20, 20%), which was not seen as often in *KRAS*-mutated or *KRAS/BRAF* wild-type adenocarcinomas. In 3 of the 4 *BRAF*-mutated colon adenocarcinomas with a signet ring cell component in our series, the signet ring cells accounted for <10% of the tumor, with 1 case containing 50% of tumor cells with signet ring cell morphology. The associations of *BRAF* mutation and both mucinous histology and signet ring histology were statistically significant ($P = 0.0002$ and 0.03 , respectively).

The histologic subtypes of the precursor polyp and any synchronous colorectal polyps, when identified, were also recorded for each colonic adenocarcinoma. The precursor polyps were most frequently adenomas in *BRAF*-mutated (3 tubular adenomas, 4 tubulovillous adenomas), *KRAS*-mutated (21 tubular adenomas, 17 tubulovillous adenomas, and 1 villous adenoma), and *KRAS/BRAF* wild-type adenocarcinomas (23 tubular adenomas, 10 tubulovillous adenomas, and 3 villous adenomas). In contrast, there was an increased frequency of synchronous sessile serrated polyps (4/20, 20%) identified in *BRAF*-mutated adenocarcinomas compared with *KRAS*-mutated (4/78, 5%) and *KRAS/BRAF* wild-type adenocarcinoma (4/83, 5%), although this did not reach statistical significance ($P = 0.06$).

Clinical Outcome Stratified by *KRAS* and *BRAF* Mutation Status

Clinical follow-up data were available for 173 patients with a median follow-up of 12 months (range, 0 to 76 mo) (Table 2). There was a statistically significant difference in survival between the 3 groups ($P < 0.0001$) (Fig. 2). Although the precision of the survival estimates was limited by the small sample size of 20 *BRAF*-mutated colonic adenocarcinomas, patients with *BRAF*-mutated adenocarcinomas had a median survival of approximately 12 months, with 54% probability of living for 1 year and a 56% chance of surviving progression-free for 1 year. Interestingly, during the follow-up period of this study, most deaths and disease recurrences in patients with *BRAF*-mutated adenocarcinomas occurred within approximately 1 year of the patient's initial surgical resection procedure, suggesting that *BRAF*-mutated adenocarcinomas have a particularly aggressive early clinical course. All patients with *BRAF*-mutated adenocarcinomas were either dead or lost to follow-up at 2 years, except 1 patient who died at 25 months. Compared with patients with *BRAF*-mutated adenocarcinomas, patients with *KRAS*-mutated adenocarcinomas had significantly improved overall survival probability (1-year, 87% and 3-year, 60%; unadjusted log-rank $P = 0.03$ and Bonferroni-adjusted log-rank $P = 0.08$) and disease-free survival probability (1-year, 95% and 3-year, 68%; unadjusted log-rank $P = 0.02$ and Bonferroni-adjusted log-rank $P = 0.05$). Similarly, compared with patients with *BRAF*-mutated adenocarcinomas, patients with *KRAS/BRAF* wild-type adenocarcinomas had significantly improved overall survival (1-year, 92% and 3-year, 65%; unadjusted log-rank $P = 0.0002$ and Bonferroni-adjusted log-rank $P = 0.0005$) and disease-free survival (1-year, 87% and 3-year, 65%; unadjusted log-rank $P = 0.02$ and Bonferroni-adjusted log-rank $P = 0.06$). No significant difference in overall or disease-free survival was identified between patients with *KRAS*-mutated and *KRAS/BRAF* wild-type adenocarcinomas.

When adjusting for tumor stage (Table 3), survival analysis assessing *KRAS* and *BRAF* mutation status demonstrated that patients with *BRAF*-mutated adenocarcinoma had a significantly poor overall survival and disease-free survival (hazard ratio 6.63, 95% CI, 2.60–16.94; and hazard ratio 6.08, 95% CI, 2.11–17.56, respectively) compared with patients with *KRAS/BRAF* wild-type adenocarcinomas. In contrast, there was no difference in overall survival or disease-free survival between patients with *KRAS*-mutated adenocarcinoma and those with *KRAS/BRAF* wild-type adenocarcinomas (Table 3).

DISCUSSION

Our results demonstrate that, in the proximal colon, *BRAF*-mutated adenocarcinomas with proficient DNA mismatch repair frequently demonstrate mucinous histology and are particularly aggressive with a significantly worse overall survival compared with *BRAF* wild-type and *KRAS*-mutated colon adenocarcinomas. The *BRAF*-mutated adenocarcinomas with proficient DNA mismatch repair identified in our study also display morphologic features typically associated with adverse behavior, including lymphatic invasion, perineural invasion, high tumor budding, and lymph node metastases.

Mucinous colonic adenocarcinoma is a frequently encountered histologic subtype of colorectal carcinoma, accounting for between 10% and 15% of colorectal cancer cases. There are many conflicting reports in the literature regarding the prognostic significance of mucinous colorectal carcinoma. In one of the earliest studies, Symonds and Viskery⁴⁴ demonstrated a decreased overall survival for mucinous colorectal carcinoma compared with nonmucinous carcinoma, although the difference in survival was most striking for mucinous carcinomas of the rectum. In subsequent studies, mucinous carcinoma has been shown to be independently associated with decreased overall survival in some^{7,37,41} but not all studies.^{6,11,14,21} Many of the studies evaluating the effect of mucinous differentiation on prognosis in colorectal carcinoma have failed to differentiate mucinous from signet ring cell carcinomas^{37,41}; the latter are generally considered to be a more aggressive colorectal carcinoma subtype. In addition, most studies include in their analysis both proximal and distal colon and rectal carcinomas, which likely have distinctly different molecular characteristics. Assessing the importance of mucinous morphology and prognosis is made much more difficult given the close association of mucinous histology and MSI-H.⁴⁵ MSI-H status is generally associated with improved overall and disease-free survival.³¹ Thus, the presence of MSI-H often confounds analysis of mucinous histology and survival in colorectal carcinoma. Of the few studies analyzing both MSI status and mucinous histology, MSI-H colorectal carcinomas had an improved survival compared with microsatellite-stable mucinous colorectal carcinomas,^{19,24} although the survival benefit was not independent of stage. Compared with these previous literature reports, we have taken several steps to eliminate the potential biases in this study that could have affected the outcome results. By restricting our analysis to colon adenocarcinomas demonstrating proficient DNA mismatch repair either by mismatch repair protein immunohistochemistry or MSI PCR analysis, we eliminated the potential confounding effect of sporadic MSI-H colorectal carcinomas that frequently harbor *BRAF* mutations^{8,48} and that are generally associated with improved overall and disease-free survival.³¹ We also limited our analysis to proximal colon adenocarcinoma given previous literature reports identifying a higher proportion of *BRAF*-

mutated colorectal adenocarcinomas in the proximal colon^{30,36} and the distinctly different pathways of carcinogenesis between the left and right colon.^{9,40} As such, we have removed the potential effects of tumor location and MSI on prognosis. The subset of *BRAF*-mutated proximal colonic adenocarcinoma with mucinous histology and dismal prognosis identified in our analysis may help to explain the poor survival of mucinous colon adenocarcinomas previously reported in some studies.

A number of studies have previously demonstrated the association of *BRAF* mutation and poor prognosis in microsatellite-stable colorectal carcinoma. Kakar et al²⁰ analyzed 83 carcinomas from both the right and left co-lons and identified 10 cases that were microsatellite stable and harbored *BRAF* mutations (5 proximal and 5 distal). *BRAF*-mutated microsatellite-stable carcinomas had significantly worse 5-year survival, and *BRAF* mutation was found to be an independent predictor of poor survival in microsatellite-stable colorectal cancer. Detailed morphologic features of *BRAF*-mutated microsatellite-stable colorectal carcinomas were not specifically reported by Kakar et al.²⁰ Samowitz et al³⁶ studied 911 colorectal carcinomas and identified 40 (5%) *BRAF*-mutated microsatellite-stable colorectal carcinomas that had a significantly worse stage-adjusted overall survival. In addition, Samowitz and colleagues reported that 10/40 (25%) *BRAF*-mutated microsatellite-stable colorectal carcinomas demonstrated mucinous histology compared with 78/685 (10.2%) *BRAF* wild-type microsatellite-stable colorectal carcinoma, similar to our results. However, in this large population-based study by Samowitz and colleagues, mucinous histology was not clearly defined, and it appears that not all cases were histologically rereviewed for the study. Furthermore, in this study, other histologic characteristics were not rigorously analyzed, and detailed comparative analysis of *BRAF*-mutated and *BRAF* wild-type tumors was not stratified by location. In addition, in the Samowitz and colleagues' cohort, proximal location was associated with decreased overall survival in their univariate analysis. As *BRAF*-mutated tumors in the study by Samowitz and colleagues were preferentially located in the proximal colon, location may have potentially biased their results. Finally, Ogino et al³⁰ identified *BRAF* mutations in 52 of 528 (10%) microsatellite-stable colon carcinomas, many of which were located in the proximal colon. In contrast to Samowitz et al and Kakar et al, Ogino et al did not identify a difference in colon cancer-specific mortality between *BRAF*-mutated and *BRAF* wild-type microsatellite-stable colorectal carcinoma. However, in their analysis, Ogino et al found that *BRAF* mutation status when coupled with CIMP status stratified patients prognostically: *BRAF*-mutated CIMP-low/negative colon cancers had a significantly worse colon cancer-specific mortality compared with *BRAF* wild-type CIMP-low/negative colon cancers. Combined with our analysis, all these literature reports suggest that presence of *BRAF* mutation is a predictor of poor survival in colon adenocarcinoma. Our study is complementary to these prior studies and enhances previous data by performing a rigorous analysis of the histopathologic findings of the clinically aggressive *BRAF*-mutated, microsatellite-stable proximal colon adenocarcinoma.

In general, signet ring carcinoma has been shown to be independently associated with decreased overall survival.^{21,25,28,32,42} In our analysis, a small subset of *BRAF*-mutated proximal colon adenocarcinoma also demonstrated focal signet ring cell morphology. Ogino et al analyzed 32 colorectal carcinomas with a signet ring component, with 9 (28%)

demonstrating *BRAF* mutations and 10 (26%) demonstrating *KRAS* mutations, indicating that mutations in the EGFR signaling pathway are not infrequent in signet ring colorectal carcinoma. However, in contrast to Ogino et al, in our series signet ring cell morphology was more often identified in *BRAF*-mutated adenocarcinomas compared with *KRAS*-mutated tumors. A number of reasons may explain the discrepancy between our results and that of Ogino et al: (1) Ogino et al included carcinomas from the colon and rectum, whereas our analysis was restricted to proximal colon adenocarcinoma; (2) Ogino et al included tumors with high levels of MSI, whereas we restricted our analysis to microsatellite-stable carcinomas. Kakar et al studied 72 signet ring colorectal carcinomas, of which 39 were right sided, with most (81%) exhibiting MSI-H. Although Kakar et al did not identify a significant survival difference between MSI-H and microsatellite-stable signet ring cell carcinomas, stratification of these tumors based on *BRAF* mutation analysis and site of disease was not performed. Overall, our results indicate that among microsatellite-stable proximal colon adenocarcinomas, *BRAF*-mutated adenocarcinomas more often display signet ring morphology compared with *KRAS/BRAF* wild-type and *KRAS*-mutated adenocarcinomas.

In summary, *BRAF*-mutated proximal colon adenocarcinomas with proficient DNA mismatch repair have a dismal prognosis and an aggressive clinical course. In addition, these tumors often display mucinous differentiation, focal signet ring histology, and other adverse histologic features such as lymphatic and perineural invasion and high tumor budding. It is important that this subset of proximal colon adenocarcinoma be recognized clinically, as death and tumor recurrence typically occur early in the clinical course of the disease. Furthermore, as targeted therapies emerge for *BRAF*-mutated tumors, early recognition of this subset of colonic adenocarcinomas may become more essential to institute appropriate treatment.

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FIGURE 1.

Microsatellite-stable proximal colonic adenocarcinomas with *BRAFV600E* mutation frequently demonstrated mucinous histology in both the primary tumor (A, $\times 100$) and lymph node metastases (B, $\times 200$), as well as lymphatic invasion (C, $\times 100$), perineural invasion (D, $\times 200$), and high tumor budding (D).

FIGURE 2.

(A) Kaplan-Meier survival curves comparing the overall survival of patients with proximal colon adenocarcinomas with proficient DNA mismatch repair harboring *KRAS* mutations, *BRAF*V600E mutation, or *KRAS* and *BRAF* wild type. Patients with *BRAF*-mutated adenocarcinomas had a significantly worse overall survival compared with *KRAS*-mutated (unadjusted log-rank $P = 0.03$) and *KRAS/BRAF* wild-type (unadjusted log-rank $P = 0.0002$) adenocarcinomas. (B) Kaplan-Meier survival curves comparing the disease-free survival of patients with proximal colon adenocarcinomas with proficient DNA mismatch repair harboring *KRAS* mutations, *BRAF*V600E mutation, or *KRAS* and *BRAF* wild type. Patients with *BRAF*-mutated adenocarcinomas had a significantly worse disease survival compared with *KRAS*-mutated (unadjusted log-rank $P = 0.02$) and *KRAS/BRAF* wild-type (unadjusted log-rank $P = 0.02$) adenocarcinomas.

TABLE 1.
Clinical, Pathologic, and Molecular Features of Proximal Colonic Adenocarcinomas With Proficient DNA Mismatch Repair

Clinical or Molecular Feature	All Cases	KRAS Mutation	BRAF Mutation	KRAS and BRAF Wild-type	P
Total number	181	78	20	83	
Sex (%)					
Male	96 (53)	44 (56)	7 (35)	44 (53)	0.23
Female	85 (47)	34 (44)	13 (65)	39 (47)	
Mean age, years (range)	67 (33–98)	68 (35–95)	71 (45–98)	65 (33–85)	0.20
Tumor stage (%)					
I	28 (15)	13 (17)	1 (5)	14 (17)	0.45
II	50 (28)	20 (26)	4 (20)	26 (31)	
III	69 (38)	30 (38)	8 (40)	31 (37)	
IV	34 (19)	15 (19)	7 (35)	12 (15)	
Tumor grade (%)					
Low	145 (80)	65 (83)	12 (60)	68 (82)	0.07
High	36 (20)	13 (17)	8 (40)	15 (18)	
Lymphatic invasion (%)	91 (50)	41 (53)	16 (80)	34 (41)	0.006
Lymph node metastasis (%)	98 (54)	43 (55)	14 (70)	41 (49)	0.02
Mean number of lymph node metastasis	2.4	2.5	4.5	1.8	0.02
Perineural invasion (%)	21 (12)	8 (10)	8 (40)	5 (6)	0.0006
Venous invasion (%)	29 (16)	12 (15)	5 (25)	12 (14)	0.50
Tumor-infiltrating lymphocytes (%)					
Absent	166 (92)	74 (95)	18 (85)	74 (97)	0.18
Present	15 (8)	4 (5)	2 (10)	9 (11)	
Crohn-like reaction (%)					
Absent	107 (59)	44 (56)	9 (45)	54 (65)	0.29
Mild	49 (27)	25 (32)	7 (35)	17 (20)	
Intense	25 (14)	9 (12)	4 (20)	12 (15)	
Tumor budding (%)					
Low	82 (45)	34 (44)	4 (20)	44 (53)	0.02
High	99 (55)	44 (56)	16 (80)	39 (47)	

Clinical or Molecular Feature	All Cases	KRAS Mutation	BRAF Mutation	KRAS and BRAF Wild-type	P
Total number	181	78	20	83	
Mucinous histology (%)					0.0002
Absent	120 (66)	53 (68)	5 (25)	62 (75)	
Present	61 (34)	25 (32)	15 (75)	21 (25)	
Focal (<50%)	42 (23)	19 (24)	9 (45)	14 (17)	
Prominent (>50%)	19 (11)	6 (8)	6 (30)	7 (8)	
Signet ring histology (%)					0.03
Absent	171 (94)	75 (96)	16 (80)	80 (95)	
Present	10 (6)	3 (4)	4 (20)	3 (5)	
Focal (<50%)	8 (5)	3 (4)	3 (15)	2 (4)	
Prominent (>50%)	2 (1)	0	1 (5)	1 (1)	
Precursor lesion (%)					0.46
Adenomatous	82 (45)	39 (50)	7 (35)	36 (43)	
Serrated	2 (1)	0	1 (5)	1 (1)	0.21
Synchronous polyp(s) (%)					0.23
Adenomatous	61 (34)	29 (37)	9 (45)	23 (28)	
Serrated	12 (7)	4 (5)	4 (20)	4 (5)	0.06

TABLE 2.
Proximal Colonic Adenocarcinomas With Proficient DNA Mismatch Repair: Long-term Follow-up According to BRAF and KRAS Mutation Status

Mutation Status	Patients	Median Follow-up in Months (range)	NED	AWD	DOD	Overall Survival (%)		Disease-free Survival (%)	
						1 y	3 y	1 y	3 y
BRAF mutation	19	8 (1–26)	7	3	9	54	*	56	*
KRAS mutation	71	12 (1–72)	52	7	12	87	60	95	68
BRAF and KRAS wild-type	83	12 (1–76)	67	9	7	92	65	87	65

* All patients with BRAF-mutated adenocarcinomas except 1 patient who died at 25 months were either dead or lost to follow up at 2 years.

AWD indicates alive with disease; DOD, dead of disease; NED, no evidence of disease.

TABLE 3.

Stage-adjusted Survival Analysis of Proximal Colonic Adenocarcinomas With Proficient DNA Mismatch Repair Stratified by KRAS and BRAF Mutation Status

Molecular Feature	Total	Hazard Ratio (95% CI)	
		Overall Survival	Disease-free Survival
KRAS and BRAF wild-type	83	1 (referent)	1 (referent)
KRAS mutation	71	1.27 (0.58–2.81)	0.88 (0.36–2.12)
BRAF mutation	19	6.63 (2.60–16.94)	6.08 (2.11–17.56)

Statistical analysis performed after adjusting for stage by grouping stages 1 to 2 and stages 3 to 4 tumors.