KRAS Codon 12 and 13 Mutations in Relation to Disease-Free Survival in BRAF–Wild-Type Stage III Colon Cancers from an Adjuvant Chemotherapy Trial (N0147 Alliance)

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Abstract

**Purpose:** We examined the prognostic impact of specific KRAS mutations in patients with stage III colon adenocarcinoma receiving adjuvant FOLFOX alone or combined with cetuximab in a phase III trial (N0147). Analysis was restricted to BRAF–wild-type tumors, because BRAF mutation was associated with poor prognosis, and BRAF and KRAS mutations are mutually exclusive.

**Experimental Design:** The seven most common KRAS mutations in codon 12 and codon 13 were examined in 2,478 BRAF–wild-type tumors. Because KRAS mutations in codon 12 (n = 779) or 13 (n = 220) were not predictive of adjuvant cetuximab benefit, study arms were pooled for analysis. Disease-free survival (DFS) was evaluated by HRs using Cox models.

**Results:** KRAS mutations in codon 12 (multivariate HR, 1.52; 95% confidence interval, CI, 1.28–1.80; P < 0.0001) or codon 13 (multivariate HR, 1.36; 95% CI, 1.04–1.77; P = 0.0248) were significantly associated with shorter DFS compared with patients with wild-type KRAS/BRAF tumors, independent of covariates. KRAS codon 12 mutations were independently associated with proficient mismatch repair (P < 0.0001), proximal tumor site (P < 0.0001), low grade, age, and sex, whereas codon 13 mutations were associated with proximal site (P < 0.0001).

**Conclusion:** KRAS mutations in either codon 12 or 13 are associated with inferior survival in patients with resected stage III colon cancer. These data highlight the importance of accurate molecular characterization and the significant role of KRAS mutations in both codons in the progression of this malignancy in the adjuvant setting. Clin Cancer Res; 20(11); 3033–43. ©2014 AACR.

Introduction

KRAS is a small G protein that acts as a transducer in the EGF receptor (EGFR) signaling pathway (1). Approximately 40% of colorectal cancers (CRC) harbor activating mutations in KRAS, making it the most commonly mutated gene in the RAS/RAF/MAPK pathway. KRAS mutations are believed to be an early event in colorectal tumorigenesis and lead to constitutive signaling and downstream activation of mitogen-activated protein kinase (MAPK)– and phosphoinositide 3-kinase (PI3K)–dependent pathways. Most (90%) KRAS mutations occur in codons 12 and 13 in the phosphate-binding loop of KRAS (1), and mutations in either codon possess transforming capacity (2, 3). In vitro evidence indicates that KRAS codon 12 mutations have greater transforming ability characterized by inhibition of apoptosis, enhanced loss of contact inhibition, and increased predisposition to anchorage-independent growth when compared with codon 13 mutations (2–4). The glycine-to-aspartate transition (p.G13D) is the most frequent codon 13 mutation in CRC. In vitro and mouse model data have showed that, although p.G12V-mutated CRC were insensitive to cetuximab, p.G13D-mutated cells were sensitive, as were KRAS wild-type cells (5).

Whereas the ability of most KRAS mutations to predict resistance to anti-EGFR therapy in patients with metastatic colorectal cancer is widely accepted, including recommendations for KRAS testing in metastatic disease (6), the prognostic impact of KRAS mutations, including in stage III disease, is uncertain (7–10). Codon 12 mutations have been associated with adverse prognosis in aggregate colorectal cancer populations of diverse disease stages (11, 12). However, recent data suggest that KRAS codon 13 mutations may not represent an aggressive phenotype or confer resistance to anti-EGFR therapy compared with wild type. In...
metastatic CRC, codon 13 (p.G13D) mutation, in contrast with those in codon 12, was associated with sensitivity to anti-EGFR therapy that was similar to wild type (5, 13), though the literature is inconsistent (14). Furthermore, recent population-based data suggest that patients with KRAS codon 13 mutations may have similarly favorable prognosis as those with KRAS wild type (11). No study to date has demonstrated that KRAS codon 13 mutations are significantly associated with worse patient survival in patients with nonmetastatic colon cancer, and highlight the important role of both codon 12 and 13 mutations in the progression of this malignancy in the adjuvant setting.

Few studies examining the prognostic impact of specific KRAS mutations in CRC have controlled for BRAF mutation as a confounder. However, the most rigorous approach to isolate the prognostic impact of KRAS is to restrict analysis to BRAF–wild-type tumors, given that BRAF and KRAS mutations are mutually exclusive (6) and that BRAF mutations are associated with adverse prognosis (7, 18, 20–24). It is also important to account for DNA mismatch repair (MMR) status, as the subset of CRCs with deficient MMR (dMMR) and microsatellite instability have a relatively low rate of KRAS mutations as compared with proficient MMR (pMMR) and microsatellite-stable tumors (25).

In this report, we determined the association of the seven most common KRAS mutations in codons 12 and 13 with disease-free survival (DFS) in prospectively collected, stage III colon adenocarcinomas from participants of a phase III trial (N0147). Patients were randomized to adjuvant 5-fluorouracil, oxaliplatin, and leucovorin (mFOLFOX6) alone or combined with cetuximab, and the addition of cetuximab to FOLFOX failed to improve DFS overall or in patients with wild-type KRAS tumors (26). The current prognostic analysis was restricted to patients whose tumors were wild type for BRAF. In this cohort, we previously reported that KRAS (all codons combined) or BRAF mutations were each associated with shorter DFS (25). In the current study, we examined KRAS mutations in codons 12 and 13 separately, with a focus on determining whether codon 13 mutations are prognostic. Our findings indicate that KRAS mutations in both codons 12 and 13 confer a worse prognosis in stage III colon cancers.

Materials and Methods

Study population

Subjects with completely resected, stage III colon adenocarcinoma (TanyN1-2M0) participated in a phase III randomized trial (North Central Cancer Treatment Group, NCCCTG N0147; 2004 to 2009) of adjuvant mFOLFOX6 alone or combined with cetuximab, which was previously described (26). Prospective and centralized KRAS mutation testing was required, although randomization was done irrespective of KRAS status in the original trial design. In August 2008, the trial was amended to restrict randomization to patients with KRAS–wild-type tumors based upon data demonstrating the predictive utility of KRAS for anti-EGFR antibody therapy (26). After amendment, eligible patients with KRAS-mutated tumors \( (n = 332) \) were treated at investigator discretion (97% received FOLFOX) and followed for disease recurrence. To avoid selection bias, the current analysis includes all randomized study patients and those with KRAS-mutated tumors who enrolled after amendment \( (n = 3,018 \text{ total}) \). Tissues were prospectively collected and required for study participation. Central pathology review was performed. Proximal tumor site included the cecum, ascending and transverse colon; distal site included the splenic flexure, descending, and sigmoid colon.

Patients initiated chemotherapy within 10 weeks of surgery. After completing protocol-specified treatment, disease recurrence was assessed every 6 months until 5 years after randomization with a physical examination, computed tomographic scan, and laboratory assessment. Follow-up colonoscopy was recommended at years 1 and 4 after resection.

The study was approved by the Institutional Review Board (IRB) of the Mayo Clinic and the NCCCTG (now part of Alliance for Clinical Trials in Oncology). Patients signed an IRB-approved consent.

KRAS and BRAF mutation

Assessment of KRAS and BRAF (NCBI Entrez Gene 673) mutational status was performed centrally at the Mayo Clinic in a Clinical Laboratory Improvement Amendments (CLIA)-compliant laboratory, using appropriate quality control procedures. Both KRAS and BRAF mutation status were determined using DNA extracted from macrodissected formalin-fixed, paraffin-embedded tumor tissue.

For KRAS, testing was performed with the DxS mutation test Kit KR-03/04 (DxS), together with the Light-Cycler 480
Table 1. Randomized clinical trials examining the prognostic impact of KRAS codon 12 and 13 mutations in colorectal cancer

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number of tumors (Total codon 12/13)</th>
<th>Percentage of total cohort</th>
<th>Tumor stage</th>
<th>Treatment</th>
<th>Multivariate HRs</th>
<th>Reference Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co.17, BOND, MABEL, EMR202600, EVEREST, BABEL, SALVAGE (5)</td>
<td>579 (260/45)</td>
<td>90%</td>
<td>CRC IV</td>
<td>BSC ± cetuximab; chemotherapy</td>
<td>c.38G&gt;A; HR, 1.82 (P = 0.053) for OS</td>
<td>BRAF/KRAS wild-type or BRAF-mutated</td>
</tr>
<tr>
<td>OPUS, CRYSTAL (13)</td>
<td>1,378 (125/83)</td>
<td>90%</td>
<td>CRC IV</td>
<td>FOLFOX or C6 cetuximab</td>
<td>c.35G&gt;T; HR, 1.22 (P = 0.16) for time to recurrence</td>
<td>BRAF/KRAS wild-type or BRAF-mutated</td>
</tr>
<tr>
<td>NSABP C07, C08 (9)</td>
<td>2,299 (229)</td>
<td>48%</td>
<td>Colon II–III</td>
<td>5FU ± oxaliplatin; bevacizumab</td>
<td>c.35G&gt;C; HR, 0.97 (P = 0.92) for relapse-free survival; c.35G&gt;T; HR, 1.09 (P = 0.64) for DFS</td>
<td>BRAF/KRAS wild-type or BRAF-mutated</td>
</tr>
<tr>
<td>PETACC-3 (18)</td>
<td>1,321 (368/102)</td>
<td>40%</td>
<td>Colon II–III</td>
<td>5FU ± irinotecan</td>
<td>c.35G&gt;A; HR, 0.98 (P = 0.91) for DFS</td>
<td>BRAF/KRAS wild-type or BRAF-mutated</td>
</tr>
<tr>
<td>CALGB 89803 (21)</td>
<td>506 (123/53)</td>
<td>40%</td>
<td>Colon III</td>
<td>5FU ± irinotecan</td>
<td>Any codon 12; HR, 1.09 (NS) for DFS</td>
<td>BRAF/KRAS wild-type or BRAF-mutated</td>
</tr>
<tr>
<td>NCCTG N0147 (Alliance); current study</td>
<td>2,478 (779/220)</td>
<td>82%</td>
<td>Colon III</td>
<td>FOLFOX ± cetuximab</td>
<td>Any codon 12; HR, 1.52 (P &lt; 0.0001) for DFS; c.38G&gt;A; HR, 1.36 (P = 0.025) for DFS</td>
<td>BRAF/KRAS wild-type only</td>
</tr>
</tbody>
</table>

Abbreviations: BSC, best supportive care; 5FU, fluorouracil; NS, not statistically significant.

aRefers to the patient reference group used for prognostic analysis.
bBSC-alone arm.
cChemotherapy-alone arms across both trials.
dData pooled across both arms.
(Roche Applied Sciences), which assesses for 7 missense point mutations: six mutations in codon 12 (c.35G>C [p. G12A, GGT>TGT], c.34G>C [p.G12R, GGT>GAT], c.35G>A [p.G12D, GGT>GAT], c.34G>T [p.G12C, GGT>GAT], c.34G>A [p.G12S, GGT>AGT], and c.35G>T [p.G12V, GGT>GAT]), and one mutation in codon 13 (c.38G>A [p. G13D, GGT>GAC]). The level of detection was set at 5%. Assessment for the BRAF c.1799T>A (p.V600E) mutation was performed using a multiplex allele-specific PCR-based assay. The PCR primers used for this assay were fluorescently labeled and included the following (wild-type forward [NEDTGATITTTGGTCTAGCTACAGT]; mutant forward [6- Fam-CAGTGATITTTGGTCTAGCTACAGT]; and reverse [GTITCCTCTAGTACTACAGCTACAGT]). Following amplification, PCR products were analyzed on an ABI 3130xl instrument (Life Technologies; Applied Biosystems) and scored for the presence or absence of the V600E variant only.

DNA mismatch repair proteins

MMR protein (MLH1, MSH2, and MSH6) expression was analyzed in formalin-fixed, paraffin-embedded tumor sections using an immunoperoxidase method (27). Monoclonal antibodies included mouse anti-human MLH1 (clone G168-15; Biocare Medical), anti-human MSH2 (clone FE11; Biocare Medical), and anti-human MSH6 (clone BC/44; Biocare Medical). MMR protein loss was defined as the absence of nuclear staining in tumor cells in the presence of positive nuclear staining in normal colonic epithelium and lymphocytes. Tumors were classified as MMR-deficient (vs. MMR-proficient) if loss of one or more MMR proteins was detected.

Statistical methods

Our primary objective was to compare survival among patients carrying any mutation in codon 12, mutated codon 13, and wild-type KRAS. The primary clinical endpoint was DFS, and a secondary endpoint was time to recurrence (TTR). DFS was defined as the time from randomization to first documented recurrence or any-cause death, whichever occurred first. TTR was defined as the time from randomization to first documented recurrence. Survival was evaluated by HR using Cox models. Kaplan–Meier methods were used to describe the distributions of DFS and TTR, which were censored at 5 years after randomization. Multivariable Cox models were adjusted for age, gender, T stage, N stage, number of examined nodes, histologic grade, performance status, primary tumor site, mismatch repair status, and treatment. Analysis of KRAS mutations included analysis of codon 12 mutations grouped together and codon 13, as well as each mutation individually. Interactions between KRAS mutation and treatment were assessed. All analyses were based on the study database frozen on September 4, 2012. Two-sided P values, with values <0.05 considered statistically significant, and 95% confidence intervals (CI) are reported. Analyses were performed using SAS version 9.2 (SAS Institute Inc.). Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center.

Results

**KRAS mutations in colon cancer**

The study population comprises patients with completely resected stage III colon cancer (n = 3,018) who received adjuvant FOLOFOX-based chemotherapy in a North American phase III clinical trial (N0147; Fig. 1). KRAS and BRAF data were available in 93.5% (2,822/3,018) of patients. Tumors with both KRAS and BRAF mutations (n = 1) or KRAS mutation in both codons 12 and 13 (n = 1) were excluded.

Figure 2A shows the frequencies and types of KRAS mutations, which are consistent with prior reports (28), and the corresponding predicted amino acid sequence alterations. KRAS codon 12 or 13 (c.38G>A [p.G13D]) mutations were detected in 35.4% (999/2,822) of tumors, with 27.6% in codon 12 and 7.8% in codon 13. Within codon 12, most (82%) mutations occurred in the second base position, and the frequency of transversions (G>C, G>T) and transitions (G>A) were similar (45% and 55%, respectively). BRAF mutation occurred in 12.2% (344/ 2,822; Fig. 2A).

**KRAS mutations and clinicopathologic characteristics**

Table 2 summarizes the baseline clinicopathologic characteristics of study subjects according to KRAS and BRAF mutation status. Compared with wild type, KRAS mutations were significantly associated with older age and female sex, primarily due to mutations in codon 12, and did not differ by T stage or number of positive nodes. Compared with KRAS wild type, codon 12 and 13 mutations were each associated with proximal (vs. distal) tumor site within the colon (P < 0.0001). Codon 12 and 13 mutations were associated with low- and high-grade histology, respectively, in primary tumors.

A low frequency of KRAS mutations was detected in dMMR compared with pMMR tumors [14% (45/318) vs. 38% (944/2,464); Table 2]. Mutations in codon 12 were significantly less frequent in dMMR tumors compared with wild type (3% vs. 8%; P < 0.0001; Table 2), and this low frequency was observed across codon 12 mutations (Fig. 2B). Deficient MMR showed a strong inverse association with KRAS codon 12 mutation (OR, 0.28; 95% CI, 0.18–0.44; P < 0.0001), independent of covariates (Supplementary Table S1). However, the frequency of dMMR was similar in KRAS codon 13 mutations and KRAS/BRAF-wild type (9% vs. 8%; P = 0.7338; Table 2).

Proximal tumor site, older age, female sex, and low grade were each significantly associated with KRAS codon 12 mutation independent of covariates (all P values <0.030; Supplementary Table S1). By contrast, only proximal site (P <0.0001) showed an independent association with KRAS codon 13 mutation compared with KRAS/BRAF-wild type (Supplementary Table S1).

Similar to KRAS mutations, BRAF mutation was associated with older age, female sex, proximal site, and dMMR; and unlike KRAS, BRAF mutation was also associated with higher T and N stage, and higher histologic grade (Table 2), as previously reported (25).
KRAS mutation and patient survival in BRAF–wild-type cases

To remove the confounding effect of BRAF mutation on the prognostic impact of KRAS mutation, we analyzed BRAF–wild-type tumors only (n = 2,478) when examining patient survival and compared KRAS-mutated/BRAF–wild-type cases with KRAS–wild-type/BRAF–wild-type cases (Fig. 1). Among the 687 DFS events, there were 353 deaths during a median follow-up of 43.2 (interquartile range, 31.0–55.3) months and 616 TTR events during a median follow-up of 42.4 (interquartile range, 30.4–55.0) months for censored cases.

As shown in Fig. 3A and Table 3 (top), patient tumors with KRAS codon 13 mutations experienced shorter DFS (univariate HR, 1.46; 95% CI, 1.13–1.89; P = 0.0035 and multivariate HR, 1.36; 95% CI, 1.04–1.77; P = 0.0248), compared with those that were wild type for KRAS and BRAF, independent of clinicopathologic variables and MMR status. KRAS codon 12 mutation was also significantly associated with worse DFS (univariate HR, 1.50; 95% CI, 1.28–1.76; P < 0.0001 and multivariate HR, 1.52; 95% CI, 1.28–1.80; P < 0.0001), compared with patients whose tumors were wild type for KRAS and BRAF. Results were similar when the full cohort was analyzed adjusting for BRAF mutation (codon 13, multivariate HR, 1.334; 95% CI, 1.003–1.773; P = 0.0474 and codon 12, multivariate HR, 1.584; 95% CI, 1.328–1.890; P < 0.0001). When TTR was analyzed as the outcome variable in the BRAF–wild-type subgroup (Fig. 3B), results were consistent both for codon 13 (univariate HR, 1.46; 95% CI, 1.11–1.92; P = 0.0064 and multivariate HR, 1.34; 95% CI, 1.01–1.78; P = 0.0446) and for codon 12 (univariate HR, 1.59; 95% CI, 1.34–1.88; P < 0.0001 and multivariate HR, 1.60; 95% CI, 1.34–1.91; P < 0.0001).

Individual KRAS mutations within codon 12 were also examined in relation to patient survival (Table 3, bottom). Each mutation was associated with worse DFS compared with KRAS/BRAF-wild type (all HR point estimates > 1). Five of the six KRAS codon 12 mutations (c.34G>A [p.G12D], c.35G>T [p.G12V], c.34G>T [p.G12C], c.35G>C [p.G12A], c.34G>C [p.G12R]) demonstrated a statistically significant association with worse DFS in univariate and multivariate analysis. Results were consistent when TTR was analyzed as the outcome (data not shown).

In an exploratory analysis, we examined the prognostic association of KRAS codon 12 or 13 mutations (vs. wild type) among BRAF–wild-type tumors within various strata, including tumor site, N stage, and MMR status. No significant modifying effect by these variables was observed (all P interaction > 0.18).

The predictive value of KRAS status for cetuximab benefit was determined among patients that enrolled before August 2008, when both KRAS-mutated and –wild-type patients were randomized to chemotherapy...
with or without cetuximab (see Materials and Methods). KRAS codon 12 or 13 mutations were not associated with differential DFS among treatment arms (any KRAS mutation vs. wild type, $P_{\text{interaction}} = 0.988$; codon 12 vs. codon 13 KRAS mutations vs. wild type, $P_{\text{interaction}} = 0.628$; Supplementary Fig. S1). Individual mutations within codon 12 were also not predictive of cetuximab benefit (Supplementary Fig. S1).

**Discussion**

We analyzed the frequency of KRAS codon 12 and 13 mutations in prospectively collected stage III colon cancers from a clinical trial of adjuvant chemotherapy. KRAS mutations were detected in 33.4% (999/2,822) of tumors, with 27.6% detected in codon 12 and 7.8% in codon 13 (c.38G>A [p.G13D]). The specific KRAS mutations identified and their relative frequencies are consistent with other studies across tumor stages (28). We also determined the association of KRAS codon 12 and 13 mutations with clinicopathologic variables and survival.

The study arms were combined for analysis because the addition of cetuximab to FOLFOX trial did not improve outcome in the parent trial, and no interaction between treatment and KRAS mutation status was observed. We
Table 2. *KRAS* codon 12 and 13 mutations in relation to clinicopathologic and molecular characteristics in stage III colon cancers (N = 2,822)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild type for <em>KRAS</em> and <em>BRAF</em> (n = 1,479)</th>
<th>Any <em>KRAS</em> mutation in codon 12 or 13 (n = 999)</th>
<th>Specific <em>KRAS</em> mutation</th>
<th>Codon 12 only (n = 779)</th>
<th>Codon 13 only (n = 220)</th>
<th><em>BRAF</em> mutation (n = 344)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
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</tr>
<tr>
<td>Median (range)</td>
<td>56 (19–84)</td>
<td>58 (22–85)</td>
<td><strong>0.0008</strong></td>
<td>58 (22–85)</td>
<td><strong>0.0002</strong></td>
<td>57 (22–82)</td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n = 1,336)</td>
<td>630 (43)</td>
<td>484 (48)</td>
<td><strong>0.0041</strong></td>
<td>387 (50)</td>
<td><strong>0.0013</strong></td>
<td>97 (44)</td>
</tr>
<tr>
<td>Male (n = 1,486)</td>
<td>849 (57)</td>
<td>515 (52)</td>
<td></td>
<td>392 (50)</td>
<td></td>
<td>123 (56)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–2 (n = 423)</td>
<td>238 (16)</td>
<td>149 (15)</td>
<td></td>
<td>111 (14)</td>
<td></td>
<td>36 (17)</td>
</tr>
<tr>
<td>T3–4 (n = 2398)</td>
<td>1,241 (84)</td>
<td>849 (85)</td>
<td><strong>0.4346</strong></td>
<td>667 (86)</td>
<td><strong>0.2545</strong></td>
<td>182 (83)</td>
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<td>Grade</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Low (n = 2,116)</td>
<td>1,145 (77)</td>
<td>792 (79)</td>
<td><strong>0.2710</strong></td>
<td>639 (82)</td>
<td><strong>0.0105</strong></td>
<td>153 (70)</td>
</tr>
<tr>
<td>High (n = 706)</td>
<td>334 (23)</td>
<td>207 (21)</td>
<td></td>
<td>140 (18)</td>
<td></td>
<td>67 (30)</td>
</tr>
<tr>
<td>Number of positive nodes</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1–3 (n = 1,650)</td>
<td>871 (59)</td>
<td>610 (61)</td>
<td><strong>0.2799</strong></td>
<td>487 (63)</td>
<td><strong>0.0944</strong></td>
<td>123 (56)</td>
</tr>
<tr>
<td>4 or more (n = 1,172)</td>
<td>608 (41)</td>
<td>389 (39)</td>
<td></td>
<td>292 (37)</td>
<td></td>
<td>97 (44)</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Proximal (n = 1,407)</td>
<td>545 (37)</td>
<td>577 (59)</td>
<td><strong>0.0001</strong></td>
<td>443 (58)</td>
<td><strong>0.0001</strong></td>
<td>134 (62)</td>
</tr>
<tr>
<td>Distal (n = 1,370)</td>
<td>914 (63)</td>
<td>402 (41)</td>
<td><strong>0.0001</strong></td>
<td>321 (42)</td>
<td><strong>0.0001</strong></td>
<td>81 (38)</td>
</tr>
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<td>Missing</td>
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<td>20</td>
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<td>15</td>
<td>5</td>
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</tr>
<tr>
<td>Mismatch repair</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Deficient (n = 318)</td>
<td>124 (8)</td>
<td>45 (5)</td>
<td><strong>0.0001</strong></td>
<td>25 (3)</td>
<td><strong>0.0001</strong></td>
<td>197 (91)</td>
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<tr>
<td>Proficient (n = 2,464)</td>
<td>1,331 (92)</td>
<td>944 (95)</td>
<td><strong>0.0001</strong></td>
<td>747 (97)</td>
<td><strong>0.0001</strong></td>
<td>189 (56)</td>
</tr>
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<td>Missing</td>
<td>24</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>6</td>
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</tr>
</tbody>
</table>

*Comparison with *KRAS/BRAF* wild type.*

restricted prognostic analysis to *BRAF*–wild-type tumors so as to control for the confounding effect of *BRAF* c.1799T>A mutations. We found that *KRAS* mutations in codons 12 or 13 (c.38G>A) were each significantly associated with worse DFS compared with *KRAS*–wild-type/*BRAF*–wild-type cases. Specifically, patients whose tumors carried *KRAS* codon 12 or 13 mutations experienced a 52% or 36% higher relative risk, respectively, of colon cancer recurrence or any-cause death that was independent of clinicopathologic variables or MMR status. Results were similar when TTR was used as the outcome variable. We emphasize that only the c.38G>A mutation was analyzed in codon 13, whereas multiple mutations within codon 12 were found that showed a consistent association with adverse outcome.

To our knowledge, our data are the first to demonstrate that *KRAS* codon 13 (c.38G>A) mutations adversely affect survival in nonmetastatic colon cancer. In both a population-based cohort and a meta-analysis using individual patient data of stage I to IV CRCs, codon 13 mutations were not prognostic, in contrast with codon 12 mutations (11, 12). In smaller studies examining CRCs of metastatic or mixed stage, nonsignificant trends were reported between codon 13 mutations and worse prognosis (13, 15, 17, 29). Furthermore, a study of 160 CRCs of varying tumor stages and treatments found that *KRAS* codon 13, but not codon 12, mutations were associated with higher S-phase fractions, increased nodal metastases, and adverse outcome compared with wild type (16). A Swedish population-based study of 525 CRCs reported that individuals with *KRAS* codon 13 (but not codon 12) mutations experienced shorter cancer-specific survival in unadjusted, but not adjusted, analysis (30). Limitations of prior studies include the inconsistent incorporation of patients with *BRAF* mutations (in the comparison group) and variable patient therapies, which can confound the interpretation of the *KRAS* prognostic data (31–33). Most prior studies included stage IV patients and had fewer codon 13 mutation patients. Of note, the adverse impact of *KRAS* codon 13 mutations on survival in our study seemed to be attenuated compared with codon 12 mutations (36% vs. 52%, respectively, higher risk of DFS). Consistent with this finding are...
with BRAF independent growth, and an increased ability to suppress apoptosis when compared with codon 13 mutants (2–4). Computational analysis of the structural implications of KRAS mutations suggests that codon 12 mutation (c.35G>A, p.G12D) may impair hydrolysis of GTP, leaving KRAS in an active GTP-bound state, to a greater degree than codon 13 mutation (c.38G>A, p.G13D) or wild-type KRAS (34). In metastatic CRCs, codon 13 mutations (p. G13D), but not codon 12 mutations, were associated with sensitivity to anti-EGFR therapy that was similar to wild-type tumors (5, 13). However, cetuximab was ineffective in our study and, therefore, KRAS mutations, including those in codon 13, did not predict outcomes from adjuvant cetuximab treatment.

Within KRAS codon 12, each of the six individual mutations showed an association with shorter DFS compared with wild-type KRAS/BRAF. Although c.35G>A (p.G12D) was most common, four other mutations (c.35G>T [p. G12V], c.34G>C [p.G12R], c.34G>T [p.G12C], and c.35G>C [p.G12A]) also demonstrated a significant association with adverse outcome that was independent of covariates and sometimes seemed to be stronger. The c.34G>A [p.G12S] mutation showed the weakest association. Codon 12 RAS mutations encoding valine (p.G12V) or arginine (p. G12R) have been reported to demonstrate stronger transforming ability and a more aggressive tumorigenic phenotype than other codon 12 mutations (35–37) and to be associated with shorter patient survival compared with wild type (11, 12). Interestingly, c.34G>C [p.G12R] demonstrated the strongest association with poor survival in both our study (HR >5 for DFS) and in a population-based cohort (HR > 3 for cancer-specific death), suggesting that this codon 12 mutation is particularly aggressive despite being

![Figure 3](https://example.com/figure3.jpg)

**Figure 3.** Prognostic impact of specific KRAS mutations in 2,478 patients with BRAF–wild-type resected stage III colon cancer. KRAS mutations in codons 12 and 13, compared with wild-type BRAF and KRAS, are shown in relation to DFS (A) and time to recurrence (B).

Laboratory data showing that KRAS codon 12 mutations display greater transforming ability, enhanced anchorage-independent growth, and an increased ability to suppress

<table>
<thead>
<tr>
<th>KRAS status</th>
<th>N (events)</th>
<th>3-year DFS rate (95% CI)</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>Any codon 12 mutation</td>
<td>779 (256)</td>
<td>68% (64%–71%)</td>
<td>1.50 (1.28–1.76)</td>
<td>&lt;0.0001</td>
<td>1.52 (1.28–1.80)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Codon 13 mutation</td>
<td>220 (71)</td>
<td>67% (60%–73%)</td>
<td>1.46 (1.13–1.89)</td>
<td>0.0035</td>
<td>1.36 (1.04–1.77)</td>
<td>0.0248</td>
</tr>
<tr>
<td>Wild typeb</td>
<td>1,479 (360)</td>
<td>77% (75%–80%)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
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<tr>
<td><strong>Model 2</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Individual codon 12 mutations</td>
<td></td>
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</tr>
<tr>
<td>c.35G&gt;A (p.G12D)</td>
<td>378 (122)</td>
<td>68% (63%–73%)</td>
<td>1.51 (1.23–1.85)</td>
<td>&lt;0.0001</td>
<td>1.53 (1.23–1.89)</td>
<td>0.0001</td>
</tr>
<tr>
<td>c.35G&gt;T (p.G12V)</td>
<td>213 (68)</td>
<td>70% (63%–76%)</td>
<td>1.38 (1.07–1.79)</td>
<td>0.0145</td>
<td>1.40 (1.07–1.82)</td>
<td>0.0139</td>
</tr>
<tr>
<td>c.34G&gt;T (p.G12C)</td>
<td>82 (30)</td>
<td>61% (50%–73%)</td>
<td>1.66 (1.14–2.41)</td>
<td>0.0078</td>
<td>1.63 (1.11–2.41)</td>
<td>0.0128</td>
</tr>
<tr>
<td>c.35G&gt;C (p.G12A)</td>
<td>49 (19)</td>
<td>63% (49%–77%)</td>
<td>1.78 (1.12–2.82)</td>
<td>0.0148</td>
<td>1.75 (1.10–2.79)</td>
<td>0.0178</td>
</tr>
<tr>
<td>c.34G&gt;A (p.G12S)</td>
<td>52 (14)</td>
<td>72% (59%–85%)</td>
<td>1.28 (0.75–2.19)</td>
<td>0.3624</td>
<td>1.37 (0.80–2.35)</td>
<td>0.2485</td>
</tr>
<tr>
<td>c.34G&gt;C (p.G12R)</td>
<td>5 (3)</td>
<td>50% (16%–99%)</td>
<td>3.81 (1.23–11.87)</td>
<td>0.0209</td>
<td>5.30 (1.69–16.64)</td>
<td>0.0043</td>
</tr>
<tr>
<td>Codon 13 mutation</td>
<td></td>
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</tr>
<tr>
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<td>0.0035</td>
<td>1.36 (1.04–1.77)</td>
<td>0.0246</td>
</tr>
<tr>
<td>Wild typeb</td>
<td>1,479 (360)</td>
<td>77% (75%–80%)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
</tbody>
</table>

**Univariate**

**Multivariatea**

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*Adapted for age, gender, T stage, N stage, number of examined nodes, grade, performance status, tumor site, mismatch repair status, treatment.

bKRAS and BRAF wild type.
rare (<1%). Our findings confirm the adverse prognostic impact of c.35G>T (p.G12V) and, consistent with prior studies, suggest that c.34G>C (p.G12R) mutations are also adverse. In addition, our findings suggest the adverse impact of lower-frequency mutations within codon 12 (c.34G>T [p.G12C], c.35G>C[p.G12A]), and c.35G>A (p.G12D) that has not been previously reported in nonmetastatic colon cancers.

In our study, tumors with KRAS codon 12 mutations had a lower frequency of deficient MMR compared with tumors with codon 13 mutation or wild type, consistent with findings from a smaller report (38). Admittedly, this difference may be related to smaller size of the codon 13 subgroup, yet the frequency of deficient MMR was consistently low across all KRAS codon 12 mutations. In addition, codon 12 mutations were associated with low-grade histology, whereas cancers with codon 13 mutations were more likely to show high-grade histology. These findings are consistent with evidence indicating that KRAS mutations may arise in unique molecular and clinical contexts, as the mutational spectrum can depend on the nature of the underlying genetic instability (38, 39). Epidemiologically, colorectal cancers with codon 12 and 13 mutations have been associated with different dietary intake patterns (40, 41). Furthermore, laboratory studies have shown that codon 12 mutations demonstrate increased β3K pathway activation (2) and a distinct metabolic phenotype that promotes resistance to apoptosis (42) compared with codon 13 mutations. We found that KRAS mutations showed a higher frequency in proximal (vs. distal) colon tumors, independent of other variables (43, 44). The distribution of KRAS codon 12 versus 13 mutations did not differ considerably by tumor subsite (data not shown). Proximal colon tumors are more likely than distal tumors to be KRAS-mutated, BRAF-mutated, hypermutated, hypermethylated, and MMR-deficient (45). The explanation for why KRAS mutations show a predilection for the proximal tumor is unknown except to invoke molecular differences based on midgut and hindgut embryology. As expected, BRAF c.1799T>A mutations were enriched in tumors with dMMR and showed clinicopathologic features in common that included proximal tumor predominance, high-grade histology, older age, and female sex (46). In the N0147 study cohort and other reports, BRAF mutations are associated with shorter patient survival rates (9, 18, 21, 25). This study is the largest to evaluate the prognostic impact of specific KRAS codons 12 and 13 in stage III colon cancer. Other strengths of this study include prospective collection of tissue specimens from a large clinical trial with meticulous collection of survival data. Systemic treatment consisted of a modern chemotherapy regimen (FOLFOX) generalizable to most stage III patients in the world. KRAS and BRAF mutation status was determined in a CLIA-certified laboratory. Limitations of the study include the fact that overall survival (OS) data have not yet matured; however, the reliability of DFS as a surrogate for OS in a stage III colon cancer population has been demonstrated by our group and others (47). We await biomarker results from PETACC-8, a phase III trial of patients with colon cancer in which the addition of cetuximab to FOLFOX did not improve DFS or OS (48). We did not examine other less common mutations in KRAS, NRAS, or HRAS; recent data suggest that 17% to 18% of patients with metastatic CRC that are wild type for KRAS codon 12 or 13 harbor additional RAS activating mutations that predict a lack of response to panitumumab (49, 50).

In conclusion, we found that KRAS mutations in codons 12 and 13 were each significantly associated with shorter DFS, compared with tumors with wild-type KRAS/BRAF. In contrast with prior reports, our data establish codon 13 mutations as being adversely associated with outcome in stage III colon cancers. KRAS mutations were significantly more frequent in proximal tumors, and codon 12 mutations were less frequent in tumors with deficient versus proficient MMR. Our findings support testing for KRAS mutations in codons 12 and 13 in stage III colon cancers, as these results provide important prognostic information.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
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Writing, review, and/or revision of the manuscript: H.H. Yoon, D. Tougeron, Q. Shi, S.R. Alberts, M.R. Mahoney, S.G. Nair, S.N. Thibodeau, R.M. Goldberg, D.J. Sargent, F.A. Sinicrope
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Tougeron, M.R. Mahoney, D.J. Sargent

Grant Support
This study was supported by an NCI Senior Scientist Award (K05CA-142885 to F.A. Sinicrope) and the NCCTG Biospecimen Resource Grant (CA-114740) from the NIH. Support for correlative studies was also provided by unrestricted funds from Bristol-Myers Squibb, ImClone Systems, Sanofi-Aventis, and Pfizer. The study was conducted as a collaborative trial of the NCCTG, Mayo Clinic and was supported in part by Public Health Service grants CA-25224 and CA-37404 from the NCI, Department of Health and Human Services. The study was also supported, in part, by grants from the NCI (CA31946) to the Alliance for Clinical Trials in Oncology and to the Alliance Statistics and Data Center (CA33601). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 15, 2013; revised March 10, 2014; accepted March 24, 2014; published OnlineFirst March 31, 2014.
Clinical Cancer Research

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KRAS Codon 12 and 13 Mutations in Relation to Disease-Free Survival in BRAF–Wild-Type Stage III Colon Cancers from an Adjuvant Chemotherapy Trial (N0147 Alliance)

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