Specific Mutations in KRAS Codons 12 and 13, and Patient Prognosis in 1075 BRAF Wild-Type Colorectal Cancers

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Abstract

Purpose: To assess prognostic roles of various KRAS oncogene mutations in colorectal cancer, BRAF mutation status must be controlled for because BRAF mutation is associated with poor prognosis, and almost all BRAF mutants are present among KRAS wild-type tumors. Taking into account experimental data supporting a greater oncogenic effect of codon 12 mutations compared with codon 13 mutations, we hypothesized that KRAS codon 12–mutated colorectal cancers might behave more aggressively than KRAS wild-type tumors and codon 13 mutants.

Experimental design: Using molecular pathological epidemiology database of 1,261 rectal and colon cancers, we examined clinical outcome and tumor biomarkers of KRAS codon 12 and 13 mutations in 1,075 BRAF wild-type cancers (i.e., controlling for BRAF status). Cox proportional hazards model was used to compute mortality HR, adjusting for potential confounders, including stage, PIK3CA mutations, microsatellite instability, CpG island methylator phenotype, and LINE-1 methylation.

Results: Compared with patients with KRAS wild-type/BRAF wild-type cancers (N = 635), those with KRAS codon 12 mutations (N = 332) experienced significantly higher colorectal cancer–specific mortality [log-rank P = 0.0001; multivariate HR, 1.30; 95% confidence interval (CI), 1.02–1.67; P = 0.037], whereas KRAS codon 13–mutated cases (N = 108) were not significantly associated with prognosis. Among the seven most common KRAS mutations, c.35G>T (p.G12V; N = 93) was associated with significantly higher colorectal cancer–specific mortality [log-rank P = 0.0007; multivariate HR, 2.00; 95% CI, 1.38–2.90, P = 0.0003] compared with KRAS wild-type/BRAF wild-type cases.

Conclusions: KRAS codon 12 mutations (in particular, c.35G>T), but not codon 13 mutations, are associated with inferior survival in BRAF wild-type colorectal cancer. Our data highlight the importance of accurate molecular characterization in colorectal cancer.

Introduction

Colorectal cancer develops through a multistep carcinogenic process with an accumulation of epigenetic and genetic changes, including KRAS mutation. Approximately 40% of colorectal cancers harbor KRAS mutations, and 90% of those mutations occur in codons 12 and 13 (1–3). In contrast to the widely accepted predictive role of KRAS mutation in identifying resistance to anti-EGFR therapy (3–8), the prognostic role of KRAS mutation in colorectal cancer remains uncertain (9–14). Recently, the differential biologic effect of various KRAS mutations in colorectal cancer was brought to light by data from De Roock and colleagues (15) showing that the c.38G>A (p.G13D) mutation was associated with benefit from cetuximab, whereas KRAS codon 12 mutations were associated with resistance to cetuximab among chemotherapy-refractory colorectal cancer patients. A search of the literature to-date reveals that several studies (16–21) have compared the prognostic roles of KRAS codon 12 mutations with those of codon 13. Nonetheless, there is a lack of agreement as to the prognostic difference between KRAS codon 12 and codon 13 mutations in colorectal cancer (Table 1).

Of note, little attention has been given to the confounding effect of BRAF mutation on the relationship between KRAS mutation and clinical outcome in colorectal cancer.
In colorectal cancer, BRAF mutation status must be controlled because BRAF-mutated cancers are associated with poorer prognosis than BRAF wild-type cases, and almost all BRAF mutants are present among KRAS wild-type tumors. However, no previous large study (with a sample size of $N \geq 300$) has controlled for the effect of BRAF mutation. We therefore tested this hypothesis by conducting a study, including over 1,000 BRAF wild-type colorectal cancers, shows that KRAS codon 12 mutations (in particular, c.35G>T), but not codon 13 mutations, are associated with inferior survival, independent of clinical, pathologic, and molecular features of colorectal cancer. Our data suggest the potential need to evaluate both BRAF mutation status and specific KRAS mutation status as prognostic biomarkers for colorectal cancer.

### Materials and Methods

#### Study population

We used the database of 2 U.S. nationwide prospective cohort studies, the Nurses’ Health Study ($N = 121,701$ women followed since 1976) and the Health Professionals Follow-up Study ($N = 51,529$ men followed since 1986; ref. 29). Every 2 years, cohort participants have been sent follow-up questionnaires to identify newly diagnosed cancers in themselves and their first-degree relatives. We collected paraffin-embedded tissue blocks from hospitals where patients underwent colorectal cancer resections (29). We collected diagnostic biopsy specimens for rectal cancer patients who received preoperative treatment, to avoid artifacts or bias introduced by treatment. Hematoxylin and eosin (HE)-stained tissue sections from all colorectal cancer cases were reviewed by a pathologist (S.O.), unaware of other data. A subset of cases ($N = 172$) were reviewed by another pathologist (T.M.), and the concordance between the 2 observers was 0.96 ($k = 0.72$; $P < 0.0001$), indicating substantial agreement. The tumor differentiation was categorized as well moderate versus poor (>50% vs. ≤50% gland formation). Initially, 1,261 colorectal cancer cases diagnosed up to 2006 were included based on the availability of tumor tissue, sequencing data for both KRAS and BRAF, and survival data (Table 2). Treatment data were not available in this study. In this study, BRAF-mutated cancers ($N = 181$) were excluded from the final total of 1,075 BRAF wild-type cases as our survival analysis study base (Fig. 1, Supplementary Table S1). Patients were observed until death or January 1, 2011, whichever came first. Death of a participant was confirmed by searching the National Death Index. Informed consent was obtained from all study subjects. This study was approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women’s Hospital.

#### Sequencing of KRAS, BRAF and PIK3CA, and MSI analysis

Resected tissue was fixed, processed, and stored according to standard protocols by hospitals across the United States, where study participants had undergone surgery. Retrieved paraffin-embedded tissue blocks were stored at room temperature before use. Tissue for analysis was macrodissected from 10-micron sections on glass slides while guided by a hematoxylin and eosin–stained section with tumor areas marked by a pathologist (S.O.). Accordingly, we extracted DNA from tumor tissue enriched with neoplastic cells, without adjacent normal tissue. DNA was stored at −20 degrees centigrade before use. We carried out PCR and pyrosequencing targeted for KRAS (codons 12 and 13; ref. 30), BRAF (codon 600; ref. 31), and PIK3CA (exons 9 and 20), as previously described (32). Pyrosequencing technology has been shown to reliably detect KRAS.
Table 1. Studies on prognostic significance of KRAS codon 12 and 13 mutations in colorectal cancer

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Authors (y)</th>
<th>No. of hospitals</th>
<th>Sample size</th>
<th>Tumor location</th>
<th>Disease stage</th>
<th>No. of KRAS mutants</th>
<th>Multivariate HR (95% CI; vs. KRAS-wild-type as a referent, unless otherwise specified)</th>
<th>Notes</th>
</tr>
</thead>
</table>
| 16   | Samowitz and colleagues (2000) | Many | 1,413 | Colon | I–IV | No | KRAS 12, 1.0 (0.8–1.2)  
KRAS 13, 1.4 (0.95–2.0)  
c.35G>A, 1.1 (0.8–1.5)  
c.35G>T, 0.8 (0.5–1.2)  
c.38G>A, 1.4 (0.95–2.0)  | Cancer-specific survival  
Adjusted by age and stage. |
| 17   | Andreyev and colleagues (2001; Meta-analysis) | <35 | 2,832 | Colon and rectum | I–IV | <900 (<395) | <297 (<139) | Overall survival  
Adjusted by age, stage, and center. |
| 18   | Bazan and colleagues (2002) | 1 | 160 | Colon and rectum | I–IV | 40 (21) | 34 (28) | Cancer-specific survival  
Cohort and stage  
Covariates were location, stage, surgical resection, nodal metastasis, tumor growth pattern, lymphovascular invasion, lymphocytic infiltration, DNA aneuploidy status, and synthesis phase fraction status. |
| 19   | Roth and colleagues (2010) | Many | 1,299 | Colon | II–III | 372 (-) | 102 (-) | Relapse-free survival  
Covariates were tumor depth, nodal metastasis, and MSI.  
Adjusted by treatment arm and stage. |
| 20   | Zlobec and colleagues (2010) | 2 | 392 | Colon and rectum | I–III | 71 (-) | 27 (-) | Cancer-specific survival  
Univariate HR for overall survival (vs. KRAS wild-type/ 
BRAF wild-type as a referent; no multivariate HR provided)  
Covariates included both BRAF mutants and BRAF wild-type tumors.  
Pathologic type, number of metastasis, and presence of metastasis (liver, lung, and peritoneum). |
| 21   | Yokota and colleagues (2011) | 1 | 229 | Colon and rectum | Advanced and recurrence | 53 (-) | 26 (-) | Yes | Covariates were age, sex, performance status, BRAF, pathologic type, number of metastasis, and presence of metastasis (liver, lung, and peritoneum). |

(Continued on the following page)
mutation with 100% analytic sensitivity and specificity, even when the proportion of mutant alleles is as low as 10% (30). We dissected tumor-only areas, maintaining neoplastic cellularity of at least 30%. Assuming no laboratory error, both positive and negative predictive values are estimated to be 100%. MSI analysis was carried out using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487; ref. 2). MSI high was defined as instability in 30% or more of the markers, and MSI-low/microsatellite stability (MSS) as instability in 0% to 29% of the markers.

Methylation analyses for CpG islands and LINE-1
Using validated bisulfite DNA treatment and real-time PCR (MethyLight), we quantified DNA methylation in 8 CIMP-specific promoters [CACNA1G, CDKN2A (p16), CRABP1, IGF2, MLH1, NEUROG1, RINX3, and SOCS1; refs. 2, 33]. CIMP high was defined as the presence of 6 of 8 or more methylated promoters, CIMP low as 1 of 5 to 6 methylated promoters, and CIMP 0 as the absence (0 of 8) of methylated promoters according to the previously established criteria (2). To accurately quantify differences in relatively high LINE-1 methylation levels, we used pyrosequencing as previously described (34, 35).

Statistical analysis
All statistical analyses were carried out by using SAS (Version 9.2, SAS Institute). All P values were 2-sided. For our main hypothesis on the prognostic significance of KRAS codon 12 mutation among BRAF wild-type cases, a P value for significance was set at P = 0.05. When we carried out multiple hypothesis testing on specific KRAS mutations (the 7 most common mutations), the P value for significance was adjusted by Bonferroni correction to P = 0.007 (= 0.05/7). When we carried out multiple hypothesis testing on associations or interactions between KRAS mutations (codon 12 or 13) and other covariates, a P value for significance was adjusted by Bonferroni correction to P = 0.0021 (= 0.05/24). For categorical data, the χ² test was done. To compare mean age and mean LINE-1 methylation level, the t test assuming equal variances was carried out.

The Kaplan–Meier method was used to assess survival time distribution, and log-rank test was used. For analyses of colorectal cancer–specific mortality, deaths as a result of other causes were censored. To control for confounding, we used multivariate Cox proportional hazards regression models. A multivariate model initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), family history of colorectal cancer in any first-degree relative (present vs. absent), tumor location (colon vs. rectum), tumor differentiation (well to moderate vs. poor), MSI (high vs. low/MSS), CIMP (high vs. low/0), PIK3CA, and LINE-1 methylation (continuous). To avoid overfitting and residual confounding, disease stage (1, IIA, IIB-C, IIIA, IIIB, IIIC, IV, or unknown) was used as a stratifying variable using the "strata" option in the SAS "proc phreg" command. A backward stepwise elimination was carried out with P =
<table>
<thead>
<tr>
<th>Clinical, pathologic, or molecular feature</th>
<th>Total No. (%)</th>
<th>KRAS wild type No. (%)</th>
<th>KRAS mutant</th>
<th>P (wild type vs. all mutants together)</th>
<th>Mutation in either codon 12 or codon 13 only</th>
<th>Mutations in both codon 12 and codon 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
<td>1,261</td>
<td>810</td>
<td>451</td>
<td>0.00049</td>
<td>0.39</td>
<td>0.0079</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>568 (45%)</td>
<td>341 (42%)</td>
<td>164 (49%)</td>
<td>0.0049</td>
<td>0.39</td>
<td>0.0079</td>
</tr>
<tr>
<td>Female</td>
<td>693 (55%)</td>
<td>469 (58%)</td>
<td>171 (51%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age (y) ± SD</td>
<td>68.5 ± 8.7</td>
<td>68.2 ± 8.7</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before 1995</td>
<td>396 (31%)</td>
<td>246 (30%)</td>
<td>105 (31%)</td>
<td>0.48</td>
<td>0.0079</td>
<td></td>
</tr>
<tr>
<td>1995 –1999</td>
<td>405 (32%)</td>
<td>260 (32%)</td>
<td>100 (30%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000 –2006</td>
<td>460 (36%)</td>
<td>304 (38%)</td>
<td>130 (39%)</td>
<td></td>
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</tr>
<tr>
<td>Family history of colorectal cancer in first degree relative(s)</td>
<td>0.35</td>
<td>0.95</td>
<td></td>
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<tr>
<td>Absent</td>
<td>1,020 (81%)</td>
<td>649 (80%)</td>
<td>275 (82%)</td>
<td>0.0001</td>
<td>0.68</td>
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<tr>
<td>Present</td>
<td>241 (19%)</td>
<td>161 (20%)</td>
<td>60 (18%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>210 (17%)</td>
<td>100 (12%)</td>
<td>82 (25%)</td>
<td>0.0001</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Ascending to transverse colon</td>
<td>374 (30%)</td>
<td>260 (33%)</td>
<td>80 (24%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenic flexure to sigmoid colon</td>
<td>388 (31%)</td>
<td>254 (32%)</td>
<td>104 (31%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>276 (22%)</td>
<td>187 (23%)</td>
<td>65 (20%)</td>
<td>0.0018</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Disease stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>300 (24%)</td>
<td>199 (25%)</td>
<td>80 (24%)</td>
<td>0.0126</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>356 (28%)</td>
<td>248 (31%)</td>
<td>77 (23%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>324 (26%)</td>
<td>195 (24%)</td>
<td>95 (28%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>166 (13%)</td>
<td>95 (12%)</td>
<td>52 (16%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>115 (9.1%)</td>
<td>73 (9.0%)</td>
<td>31 (9.3%)</td>
<td>0.0018</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Well to moderate</td>
<td>1,132 (90%)</td>
<td>711 (88%)</td>
<td>315 (85%)</td>
<td>0.0001</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>122 (9.7%)</td>
<td>94 (12%)</td>
<td>18 (5.4%)</td>
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<td></td>
</tr>
<tr>
<td>MSI status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI low/MSS</td>
<td>1,047 (85%)</td>
<td>625 (79%)</td>
<td>315 (85%)</td>
<td>&lt;0.0001</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>MSI high</td>
<td>190 (15%)</td>
<td>166 (21%)</td>
<td>17 (5.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP 0</td>
<td>518 (44%)</td>
<td>335 (45%)</td>
<td>140 (44%)</td>
<td>0.0001</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>CIMP low</td>
<td>456 (39%)</td>
<td>242 (32%)</td>
<td>154 (49%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP high</td>
<td>203 (17%)</td>
<td>174 (23%)</td>
<td>21 (6.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF mutation status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1,080 (86%)</td>
<td>635 (78%)</td>
<td>332 (99%)</td>
<td>&lt;0.0001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>181 (14%)</td>
<td>175 (22%)</td>
<td>3 (0.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIK3CA mutation status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>972 (84%)</td>
<td>656 (88%)</td>
<td>240 (77%)</td>
<td>&lt;0.0001</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>188 (16%)</td>
<td>88 (12%)</td>
<td>72 (23%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean LINE-1 methylation level (%) ± SD</td>
<td>62.7 ± 9.4</td>
<td>62.8 ± 9.7</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: (%) indicates the proportion of cases with a specific clinical, pathologic, or molecular feature among each KRAS mutation status group. A P value for significance was adjusted for multiple hypothesis testing to P = 0.05/24 = 0.0021. Thus, a P value between 0.05 and 0.0021 should be regarded as of borderline significance.

Abbreviations: CIMP, CpG island methylator phenotype; MSS, microsatellite stable.
Results

**KRAS mutation status in colorectal cancer**

Among 1,261 patients with incident colorectal cancer in the 2 U.S. nationwide prospective cohort studies, we detected KRAS codon 12 and/or 13 mutations in 451 (36%) patients; 335 in codon 12 only, 110 in codon 13 only, and 6 in both codons 12 and 13. For KRAS codon 12 mutations, we identified 161 cases with c.35G>A (p.G12D, codon 12 GGT>GAT), 95 cases with c.35G>T (p.G12V, codon 12 GGT>GT), 44 cases with c.34G>T (p.G12C, codon 12 GGT>TGT), 20 cases with c.35G>C (p.G12A, codon 12 GGT>GCT), 12 cases with c.34G>A (p.G12S, codon 12 GGT>AGT), 8 cases with c.34G>C (p.G12R, codon 12 GGT>GAC), and one case with c.35_36delinsCA (p.G12A, codon 12 GGT>GCA). In codon 13, we identified 110 cases with c.38G>A (p.G13D, codon 13 GGC>GAC), 3 cases with c.37G>T (p.G13C, codon 13 GGC>TGC), 2 cases with c.38G>T (p.G13V, codon 13 GGC>GTC), and one case with c.37G>C (p.G13R, codon 13 GGC>GGC).

Table 2 summarizes the baseline characteristics of study subjects (N = 1,261) according to KRAS mutation status. There was no significant difference in any of the features examined between KRAS codon 12 and codon 13 mutants. Supplementary Table S1 summarizes the baseline characteristics of BRAF wild-type cases (N = 1075) that were used for subsequent survival analyses.

**KRAS mutation status and patient survival in BRAF wild-type cases**

When assessing the prognostic effect of KRAS mutation, it is necessary to consider a confounding effect of BRAF mutation because BRAF mutation is associated with poorer prognosis (10, 11, 19, 21–23) and inversely associated with KRAS mutation (Table 2). To assess prognostic roles of various KRAS mutations, independent of BRAF mutation status, we used BRAF wild-type tumors only and compared KRAS-mutated BRAF wild-type cases to KRAS wild-type/BRAF wild-type cases. Thus, among the 1,261 patients with KRAS and BRAF data, 181 BRAF-mutated cases were excluded (Fig. 1). Within the remaining 1,080 cases, 5 cases with KRAS mutations in both codons 12 and 13 were excluded to analyze the prognostic effect of KRAS codon 12 mutation separately from that of codon 13 mutation. As a result, a total of 1,075 BRAF wild-type cases were used for survival analyses (Fig. 1, Supplementary Table S1). There were 512 deaths, including 299 colorectal cancer-specific deaths, during a median follow-up of 11.7 years (interquartile range, 8.3–16.1 years) for censored cases.

The 5-year colorectal cancer–specific survival probabilities were 81.5% for patients with KRAS wild-type/BRAF wild-type, 68.8% for those with KRAS codon 12 mutations (with wild-type BRAF), and 75.3% for those with KRAS codon 13 mutations (with wild-type BRAF; Fig. 2 Panels A and B). Compared with patients with KRAS wild-type/BRAF wild-type cancers, those with KRAS codon 12 mutations experienced a significant increase in colorectal cancer–specific mortality in Kaplan–Meier analysis (log-rank P = 0.0001) and in Cox regression analysis [univariate HR, 1.68, 95% confidence interval (CI), 1.32–2.14, P < 0.0001; multivariate HR, 1.30; 95% CI, 1.02–1.67, P = 0.037; Table 3]. In contrast, compared with KRAS wild-type/BRAF wild-type cases, patients with KRAS codon 13 mutations did not experience any significant reduction in survival (Table 3).
Among the 7 most common KRAS codon 12 and 13 mutations, c.35G>T (p.G12V; N = 93) was associated with significantly higher colorectal cancer-specific mortality (log-rank P = 0.0007; multivariate HR, 2.00; 95% CI, 1.38–2.90, P = 0.0003) compared with KRAS wild-type/BRAF wild-type (Fig. 2 Panels C–D, Table 4). In addition, c.34G>C (p.G12R; N = 8) was associated with higher colorectal cancer-specific mortality (univariate HR, 4.22; 95% CI, 1.72–10.4, P = 0.0017; multivariate HR, 3.39; 95% CI, 1.28–9.00, P = 0.014) compared with KRAS wild-type/BRAF wild-type. Nonetheless, due to the lower statistical power, as well as multiple hypothesis testing (requiring an adjusted stringent significance level at P = 0.05/7 = 0.007), our findings from mutation-specific survival analyses required validation in independent datasets. Analyses excluding the cases with unknown disease stage (N = 110), yielded similar results (Supplementary Table S2).

To assess the impact of confounding by BRAF mutation, we repeated the above survival analyses including BRAF-mutated cases, most of which were included in the KRAS wild-type group. A total case number for this additional analysis was 1,255, including 180 BRAF-mutated cases. Compared with KRAS wild-type cases, KRAS codon 12 mutations were not significantly associated with colorectal cancer-specific mortality in multivariate analysis, and the HR effect estimate was substantially attenuated.
erably attenuated. (p.G12R) and c.35G＞wild-type cases, the HR effect estimates for c.34G＞codon 12 and 13 mutations, compared with KRAS (Supplementary Table S3). Among the 7 most common KRAS codon 12 and 13 mutations, compared with KRAS wild-type cases, the HR effect estimates for c.34G＞KRAS BRAF Total N Colorectal cancer–specific mortality Overall mortality No. of HR (95% CI) HR (95% CI) events Univariate Multivariate Univariate Multivariate events Univariate Multivariate
Wild type Wild type 635 149 1 (referent) 1 (referent) 275 1 (referent) 1 (referent)
All codon 12 mutants Wild type 332 119 1.68 (1.32–2.14) 1.30 (1.02–1.67) 183 1.46 (1.21–1.77) 1.24 (1.02–1.51)
c.34G＞wild-type cases
All codon 13 mutants Wild type 108 31 1.25 (0.85–1.84) 0.86 (0.58–1.27) 54 1.17 (0.87–1.57) 0.96 (0.71–1.30)

NOTE: We tested the specific study hypothesis on the prognostic role of KRAS codon 12 mutations, among BRAF wild-type cases. Thus, a P value for significance was set at P = 0.05. The multivariate, stage-stratified Cox regression model initially included age, sex, year of diagnosis, tumor location, tumor differentiation, family history of colorectal cancer, MSI, CpG island methylator phenotype, PIK3CA, and LINE-1 methylation. A backward stepwise elimination with a threshold of P = 0.20 was used to select variables in the final model. Abbreviation: NS, not significant.

KRAS mutation status and mortality in strata of other variables
As exploratory analyses, we examined the prognostic association of KRAS codon 12 and 13 mutation status among BRAF wild-type tumors in various strata, including...
disease stage, sex, age, family history of colorectal cancer, tumor location, tumor differentiation, MSI, CIMP, PIK3CA, and LINE-1 methylation. We did not observe considerable or significant modifying effect by any of these variables on KRAS codon 12 or 13 mutation [all \( p_{\text{interaction}} > 0.02 \); given multiple hypothesis testing, a statistical significance level was adjusted to \( p_{\text{interaction}} = 0.0021 \)].

**Discussion**

We conducted this study to assess whether KRAS codon 12–mutated tumors represent a more aggressive subtype as compared with either KRAS codon 13–mutated tumors or KRAS wild-type tumors, within a group of BRAF wild-type tumors (i.e., controlling for BRAF mutation status). We showed that KRAS codon 12 mutations, but not codon 13 mutations, were associated with significantly higher mortality compared with KRAS wild-type/BRAF wild-type cases. In particular, c.35G>T (p.G12V) mutation was associated with the highest colorectal cancer–specific mortality (multivariate HR, 2.00; 95% CI, 1.38–2.90, \( P = 0.0003 \)). Our data are consistent with previous laboratory studies (24, 25), suggesting that the presence of a mutation in KRAS codon 12 confers substantially greater oncogenic potential as compared with codon 13 mutation. Our data are also consistent with a recent study that showed that KRAS codon 12 mutations, but not codon 13 mutations, conferred resistance to cetuximab in advanced colorectal cancer (15).

Detection of somatic molecular aberrations and tumor molecular classification are increasingly important in colorectal cancer (36–39). We used pyrosequencing technology, which has been shown to be more sensitive than Sanger sequencing in detecting KRAS mutations in paraffin-embedded archival tissue (30, 40, 41). Pyrosequencing is a sensitive sequencing assay and can reliably detect mutant alleles of low abundance (10% mutant) among wild-type alleles, which is a common situation in solid tumors (30, 40, 41).

To the best of our knowledge based on the literature search in Pubmed, this is the first study to address the prognostic difference between KRAS codon 12 and codon 13 mutations in more than 1,000 of BRAF wild-type colorectal cancers (i.e., controlling for BRAF mutation status). Although several previous studies have distinguished between the prognostic associations of KRAS mutations in codon 12 and codon 13 (Table 1; refs. 16–21), none of the large studies (with a sample size \( N \geq 300 \); refs. 16, 17, 19, 20) controlled for BRAF mutation status in their analyses, and the results are conflicting. Given the consistent significant negative prognostic impact of BRAF mutations on patient survival (10, 11, 19, 21–23), and its association with KRAS wild-type tumors, the presence of patients harboring BRAF-mutated tumors within a KRAS wild-type control group would attenuate any negative prognostic effect associated with KRAS mutation status. Therefore, BRAF mutation status must be controlled to assess the precise oncogenic effect of KRAS mutation status. A simple way of controlling for BRAF mutation status is to examine the prognostic role of KRAS mutation in BRAF wild-type tumors. Indeed, BRAF mutation confounded and attenuated the negative prognostic effects of KRAS mutations in this study.

Regulation of RAS involves binding of GTP, which activates the protein. Activation of RAS enables high affinity interactions with downstream effectors such as RAF-MAPK and phosphoinositide 3-kinase. Consequently, slow intrinsic GTPase activity leads to RAS functional inactivation. This on and off switch regulation is tightly controlled by ARHGAP (Rho-GTPase activating proteins) and RAPGEF (Rap guanine-nucleotide exchange factors; ref. 42). Interestingly, RAS mutants are resistant to ARHGAP-mediated GTPase activation, leading to elevated cellular levels of RAS-GTP (42). Guerro and colleagues (24) found that KRAS codon 12 mutation, by altering the threshold for induction of apoptosis, confers a more aggressive tumor phenotype than codon 13 mutation. This suggests that codon 12 mutation results in greater resistance to ARHGAP-mediated GTPase activation than codon 13 mutation (24).

Consequently, codon 12–mutated RAS theoretically remains in an active GTP-bound state longer than codon 13–mutated or wild-type RAS. Experimental data suggest that, among the many different KRAS codon 12 mutations, c.34G>C (p.G12R) and c.35G>T (p.G12V) mutations confer more potent transforming ability than other KRAS mutations, including c.34G>A (p.G12S), c.34G>T (p.G12C), c.35G>A (p.G12D), and c.35G>C (p.G12A; ref. 43). Moreover, the GTPase activity of c.34G>C (p.G12R) and c.35G>T (p.G12V) mutants is lower than that of other KRAS mutations (25, 44). These experimental data are consistent with our observations that KRAS codon 12 mutation, especially c.34G>C (p.G12R) and c.35G>T (p.G12V), might be associated with more aggressive tumor behavior. Our findings underscore the importance of our awareness that different mutations (even in a single gene) may contribute to different tumor characteristics and support the unique tumor principle (45–47).

Limitations of this study include the lack of data on cancer recurrence. Chemotherapeutic and surgical interventions have a significant impact on disease progression in metastatic colorectal cancer. We cannot exclude the possibility that there may have been an imbalance in the use of therapeutic interventions between subgroups. KRAS mutation status has recently become an important biomarker when selecting chemotherapeutic agents for colorectal cancer therapy (6–8, 48). Given that we could not control for use of EGFR inhibitors, such as cetuximab and panitumumab, bias may have arisen through selective use of these agents within the study group. Nonetheless, it seems unlikely that chemotherapy use or regimen differed substantially by tumor KRAS mutation status, as a vast majority of cases were diagnosed in 1990s to early 2000s, before 2006, when KRAS mutation emerged as a predictive biomarker in stage IV colorectal cancer. In addition, our molecular data were not available for patients or clinicians for treatment decision-making. Another weakness of this study is the absence of data on cancer recurrence, and, as a result, disease-free survival was not an available outcome measurement in
these cohorts. Because the median survival of metastatic colorectal cancer patients was 10 to 12 months during the time period of this study (49), we believe that colorectal cancer–specific survival is a reasonably robust surrogate for cancer-specific outcomes. In fact, disease-free survival has been shown to be highly correlated with overall survival (50).

Strengths of this study include the use of data from 2 U.S. nationwide prospective cohort studies. Information on disease staging, family history of cancer, and other clinicopathologic and tumor molecular data was prospectively integrated into the molecular pathologic epidemiology database (26–28). Cohort participants who were diagnosed with colorectal cancer were presented and treated at hospitals throughout the United States, and thus more representative colorectal cancers in the general U.S. population than are patients in single or few academic medical centers. Finally, by virtue of our molecular pathologic epidemiology (26–28) database, we assessed the effects of KRAS codon 12 and 13 mutations independent of various clinicopathologic features and other critical molecular events such as BRAF and PIK3CA mutations, MSI, CIMP, and LINE-1 hypomethylation, all of which have been associated with colorectal cancer prognosis (2, 34).

In conclusion, our study of more than 1,000 colorectal cancers has shown that KRAS codon 12 mutation (in particular, c.35G>T, p.G12V), but not codon 13 mutation, is associated with worse prognosis in BRAF wild-type colorectal cancers. Different mutations in a single gene may have distinct biologic effects and clinical implications (47). Because, controlling for BRAF status, KRAS codon 12 mutations contribute to poor prognosis in colorectal cancer, it might be prudent to control for mutations in BRAF and KRAS in the study arms of future clinical trials.

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No potential conflicts of interest were disclosed. The content is solely the responsibility of the authors and does not necessarily represent the official views of NCI or NIH.

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