**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.
Almost all BRAF-mutated colorectal cancers are present within the group of KRAS wild-type cancers. Compared with BRAF wild-type cases, BRAF mutation has been associated with poorer prognosis in several studies (10, 11, 19, 21–23); hence, it is impossible to clarify the exact prognostic roles of KRAS mutations in colorectal cancer without controlling for BRAF mutation. Importantly, none of the previous large studies (with a sample size of N ≥ 300; refs. 16, 17, 19, 20) controlled for the potential confounding effect of BRAF mutation, whereas only one smaller study (N = 229) assessed BRAF status (ref. 21; Table 1). One way of controlling for BRAF mutation is to examine the prognostic significance of KRAS mutation in BRAF wild-type colorectal cancers. Considering experimental data (24, 25) supporting a greater oncogenic effect of KRAS codon 12 mutations than codon 13 mutations, we hypothesized that KRAS codon 12–mutated colorectal cancer might behave more aggressively than codon 13 mutations and KRAS wild-type tumors.

We therefore tested this hypothesis by conducting a study on the prognostic roles of KRAS codon 12 and 13 mutations using 1,261 colorectal cancers within 2 U.S. nationwide prospective cohort studies, in which there were 1,075 BRAF wild-type cancers. Because our molecular pathologic epidemiology (26–28) pool of included tumor molecular variables, including microsatellite instability (MSI), CpG island methylator phenotype (CIMP), BRAF and PIK3CA mutations, and LINE-1 methylation, we could evaluate the prognostic role of KRAS codon 12 and 13 mutations independent of other potential molecular confounders. Our findings raise a possible need for tumor subtyping based on specific KRAS and BRAF oncogene mutations in colorectal cancer.
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>
<tbody>
<tr>
<td>16</td>
<td>Samowit and colleagues (2000)</td>
<td>Many</td>
<td>1,413</td>
<td>Colon</td>
<td>I–IV</td>
<td>353 (–) 100 (–)</td>
<td>No</td>
<td>Cancer-specific survival</td>
</tr>
<tr>
<td>17</td>
<td>Andreyev and colleagues (2001; Meta-analysis)</td>
<td>&lt;35</td>
<td>2,832</td>
<td>Colon and rectum</td>
<td>I–IV</td>
<td>&lt;900 (&lt;395) 297 (&lt;139)</td>
<td>No</td>
<td>Overall survival</td>
</tr>
<tr>
<td>18</td>
<td>Bazan and colleagues (2002)</td>
<td>1</td>
<td>160</td>
<td>Colon and rectum</td>
<td>I–IV</td>
<td>40 (21) 34 (28)</td>
<td>No</td>
<td>Cancer-specific survival</td>
</tr>
<tr>
<td>19</td>
<td>Roth and colleagues (2010)</td>
<td>Many</td>
<td>1,299</td>
<td>Colon</td>
<td>II–III</td>
<td>372 (–) 102 (–)</td>
<td>No</td>
<td>Relapse-free survival</td>
</tr>
<tr>
<td>20</td>
<td>Zlobec and colleagues (2010)</td>
<td>2</td>
<td>392</td>
<td>Colon and rectum</td>
<td>I–III</td>
<td>71 (–) 27 (–)</td>
<td>No</td>
<td>Cancer-specific survival</td>
</tr>
<tr>
<td>21</td>
<td>Yokota and colleagues (2011)</td>
<td>1</td>
<td>229</td>
<td>Colon and rectum</td>
<td>Advanced and recurrence</td>
<td>53 (–) 26 (–)</td>
<td>Yes</td>
<td>Univariate HR for overall survival</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Table 1. Studies on prognostic significance of KRAS codon 12 and 13 mutations in colorectal cancer (Cont’d)

<table>
<thead>
<tr>
<th>Ref. Authors (y)</th>
<th>Disease stage</th>
<th>Sample size</th>
<th>Tumor location</th>
<th>No. of hospitals (current study)</th>
<th>No. of KRAS mutants (95% CI)</th>
<th>Mutivariate HR (BRAF wild-type as a referent)</th>
<th>P value for significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imamura et al.</td>
<td>I–IV</td>
<td>332 (119)</td>
<td>Colon and rectum</td>
<td>Many</td>
<td>108 (31)</td>
<td>Codon 12, 1.28 (0.74–2.19) Codon 13, 2.03 (1.10–3.74)</td>
<td>0.0007 (0.05/7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cancer-specific survival adjusted by stage: covariates (age, sex, year of diagnosis, tumor location, family history of colorectal cancer)</td>
<td>0.0007 (0.05/7)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Cancer-specific survival adjusted by stage: covariates (age, sex, year of diagnosis, tumor location, family history of colorectal cancer)</td>
<td>0.0007 (0.05/7)</td>
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<td></td>
<td>Cancer-specific survival adjusted by stage: covariates (age, sex, year of diagnosis, tumor location, family history of colorectal cancer)</td>
<td>0.0007 (0.05/7)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Cancer-specific survival adjusted by stage: covariates (age, sex, year of diagnosis, tumor location, family history of colorectal cancer)</td>
<td>0.0007 (0.05/7)</td>
</tr>
</tbody>
</table>

**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 [CACA1G, CDKN2A (p16), CRABP1, IGF2, MLH1, NEUROGI1, RUNX3, 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 8 to 5 of 8 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 \( \chi^2 \) 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 (I, IIA, IIB-C, IIIA, IIIB, IIC, 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>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>335</td>
<td>110</td>
<td>6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>0.0049</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>568 (45%)</td>
<td>341 (42%)</td>
<td>164 (49%)</td>
<td>59 (54%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Female</td>
<td>693 (55%)</td>
<td>469 (58%)</td>
<td>171 (51%)</td>
<td>51 (46%)</td>
<td>2 (33%)</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>69.4 ± 8.6</td>
<td>0.044</td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td></td>
<td></td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before 1995</td>
<td>396 (31%)</td>
<td>246 (30%)</td>
<td>105 (31%)</td>
<td></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>
</tr>
<tr>
<td>2000 – 2006</td>
<td>460 (36%)</td>
<td>304 (38%)</td>
<td>130 (39%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of colorectal cancer in first degree relative(s)</td>
<td></td>
<td></td>
<td>0.35</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1,020 (81%)</td>
<td>649 (80%)</td>
<td>275 (82%)</td>
<td>90 (82%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Present</td>
<td>241 (19%)</td>
<td>161 (20%)</td>
<td>60 (18%)</td>
<td>20 (18%)</td>
<td>0</td>
</tr>
<tr>
<td>Tumor location</td>
<td>&lt;0.0001</td>
<td>0.68</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>27 (25%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>Ascending to transverse colon</td>
<td>374 (30%)</td>
<td>260 (33%)</td>
<td>80 (24%)</td>
<td>32 (29%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Splenic flexure to sigmoid colon</td>
<td>388 (31%)</td>
<td>254 (32%)</td>
<td>104 (31%)</td>
<td>29 (26%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>276 (22%)</td>
<td>187 (23%)</td>
<td>65 (20%)</td>
<td>22 (20%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Disease stage</td>
<td></td>
<td></td>
<td>0.026</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>300 (24%)</td>
<td>199 (25%)</td>
<td>80 (24%)</td>
<td>20 (18%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>II</td>
<td>356 (28%)</td>
<td>248 (31%)</td>
<td>77 (23%)</td>
<td>30 (27%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>III</td>
<td>324 (26%)</td>
<td>195 (24%)</td>
<td>95 (28%)</td>
<td>30 (27%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>IV</td>
<td>166 (13%)</td>
<td>95 (12%)</td>
<td>52 (16%)</td>
<td>19 (17%)</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>115 (9.1%)</td>
<td>73 (9.0%)</td>
<td>31 (9.3%)</td>
<td>11 (10%)</td>
<td>0</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td>0.0018</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Well to moderate</td>
<td>1,132 (90%)</td>
<td>711 (88%)</td>
<td>315 (95%)</td>
<td>101 (92%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>Poor</td>
<td>122 (9.7%)</td>
<td>124 (14%)</td>
<td>18 (5.4%)</td>
<td></td>
<td>9 (8.2%)</td>
</tr>
<tr>
<td>MSI status</td>
<td>&lt;0.0001</td>
<td>0.86</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 (95%)</td>
<td>102 (94%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>MSI high</td>
<td>190 (15%)</td>
<td>166 (21%)</td>
<td>17 (5.1%)</td>
<td>6 (5.6%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>CIMP status</td>
<td>&lt;0.0001</td>
<td>0.40</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>39 (37%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>CIMP low</td>
<td>456 (39%)</td>
<td>242 (32%)</td>
<td>154 (49%)</td>
<td>59 (56%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>CIMP high</td>
<td>203 (17%)</td>
<td>174 (23%)</td>
<td>21 (6.7%)</td>
<td>7 (6.7%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>BRAF mutation status</td>
<td>&lt;0.0001</td>
<td>0.43</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>108 (98%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>Mutant</td>
<td>181 (14%)</td>
<td>175 (22%)</td>
<td>3 (0.9%)</td>
<td>2 (1.8%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>PIK3CA mutation status</td>
<td>&lt;0.0001</td>
<td>0.48</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>72 (73%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Mutant</td>
<td>188 (16%)</td>
<td>88 (12%)</td>
<td>72 (23%)</td>
<td>26 (26%)</td>
<td>2 (33%)</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>62.8 ± 9.1</td>
<td>0.15</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.
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cases) in a multivariate Cox model.

and another variable of interest (without data-missing
tumor differentiation, MSI, CIMP, 
history of colorectal cancer, tumor location, disease stage, 
other covariates (including sex, age at diagnosis, family
interaction between
tality and overall mortality). We also tested for potential
time (all

KRAS dependent variables, which were the cross-products of the
of hazards assumption was satisfied by evaluating time-
stantially alter results (data not shown). The proportionality
missing information in any of the covariates did not sub-
exclude overfitting. We confirmed that excluding cases with
those cases in the majority category of a given covariate to

mutations in both codons 12 and 13 (2.0%), CIMP (7.2%), and
[figure: Flow chart of this study. BRAF-mutated cases (N = 181) were
excluded from survival analysis to assess a prognostic role of KRAS
mutation in BRAF-wild-type tumors. In addition, cases with KRAS
mutations in both codons 12 and 13 (N = 5) were excluded, to assess a
prognostic effect of KRAS codon 12 mutations separately from that of
KRAS codon 13 mutations.

0.20 as a threshold to avoid overfitting. For cases with
missing information in any of the categorical covariates
[tumor location (0.5%), tumor differentiation (0.7%), MSI
(2.0%), CIMP (7.2%), and PIK3CA (8.5%)], we included
those cases in the majority category of a given covariate to
avoid overfitting. We confirmed that excluding cases with
missing information in any of the covariates did not subst-
entially alter results (data not shown). The proportionality
of hazards assumption was satisfied by evaluating time-
dependent variables, which were the cross-products of the
KRAS indicator variables (codon 12 mutant and codon 13
mutant; vs. KRAS wild-type/BRAF wild-type) and survival
time (all P values >0.14 for colorectal cancer–specific mor-
tality and overall mortality). We also tested for potential
interaction between KRAS mutation status and each of
the other covariates (including sex, age at diagnosis, family
history of colorectal cancer, tumor location, disease stage,
tumor differentiation, MSI, CIMP, PIK3CA, and LINE-1
methylation). An interaction was assessed by the Wald test
on the cross-product of each of the KRAS indicator variables
and another variable of interest (without data-missing
cases) in a multivariate Cox model.

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>GTT), 44 cases with c.34G>T (p.G12C,
codon 12 GGT>GTT), 20 cases with c.35G>C (p.G12A,
codon 12 GGT>GCT), 12 cases with c.34G>A (p.G12S,
codon 12 GGT>GTT), 8 cases with c.34G>C (p.G12R,
codon 12 GGT>GCT), 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

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 charac-
teristics 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 asso-
ciated with KRAS mutation (Table 2). To assess prognos-
tic 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, Supplemen-
tary Table S1). There were 512 deaths, including 299
colorectal cancer–specific deaths, during a median fol-
low-up of 11.7 years (interquartile range, 8.3–16.1 years)
for censored cases.

The 5-year colorectal cancer–specific survival probabili-
ties 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–spe-
cific 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 sur-
vival (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.

Figure 2. Kaplan–Meier curves of BRAF wild-type colorectal cancer patients according to KRAS mutation status. KRAS-mutated BRAF wild-type cases were compared with KRAS wild-type/BRAF wild-type cases to assess a prognostic role of KRAS mutation independent of BRAF mutation status. Table indicates the number of patients who were alive and at risk of death at each time point after diagnosis of colorectal cancer. A, colorectal cancer-specific survival according to KRAS codon 12 or 13 mutation status. B, overall survival according to KRAS codon 12 or 13 mutation status. C, colorectal cancer-specific survival according to KRAS c.35G>T (p.G12V) or c.34G>C (p.G12R) mutation status. D, overall survival according to KRAS c.35G>T (p.G12V) or c.34G>C (p.G12R) mutation status.
erably attenuated.

(p.G12R) and c.35G>T mutations were considered wild-type cases, the HR effect estimates for c.34G>C (p.G12S) and c.35G>T (p.G12V) mutations were considerably attenuated.

(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>C (p.G12R) and c.35G>T (p.G12V) mutations were considerably attenuated.

**Table 3. Colorectal cancer patient mortality according to KRAS mutation status in 1075 BRAF wild-type cases**

<table>
<thead>
<tr>
<th>KRAS</th>
<th>BRAF</th>
<th>Total N</th>
<th>No. of events</th>
<th>Univariate HR (95% CI)</th>
<th>Multivariate HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>635</td>
<td>149</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>All codon 12 mutants</td>
<td>Wild type</td>
<td>332</td>
<td>119</td>
<td>1.68 (1.32–2.14)</td>
<td>1.30 (1.02–1.67)</td>
</tr>
<tr>
<td>All codon 13 mutants</td>
<td>Wild type</td>
<td>108</td>
<td>31</td>
<td>1.25 (0.85–1.84)</td>
<td>0.86 (0.58–1.27)</td>
</tr>
</tbody>
</table>

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.

**Table 4. Colorectal cancer patient mortality among the 7 most common KRAS codon 12–13 mutations in 1075 BRAF wild-type cases**

<table>
<thead>
<tr>
<th>KRAS</th>
<th>BRAF</th>
<th>Total N</th>
<th>No. of events</th>
<th>Univariate HR (95% CI)</th>
<th>Multivariate HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>635</td>
<td>149</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>c.34G&gt;A (p.G12S)</td>
<td>Wild type</td>
<td>12</td>
<td>6</td>
<td>2.61 (1.15–5.91)</td>
<td>1.03 (0.44–2.44)</td>
</tr>
<tr>
<td>c.34G&gt;C (p.G12R)</td>
<td>Wild type</td>
<td>8</td>
<td>5</td>
<td>4.22 (1.72–10.4)</td>
<td>3.39 (1.28–9.00)</td>
</tr>
<tr>
<td>c.34G&gt;T (p.G12T)</td>
<td>Wild type</td>
<td>44</td>
<td>16</td>
<td>1.70 (1.01–2.85)</td>
<td>1.56 (0.92–2.65)</td>
</tr>
<tr>
<td>c.35G&gt;A (p.G12D)</td>
<td>Wild type</td>
<td>155</td>
<td>49</td>
<td>1.47 (1.07–2.04)</td>
<td>1.08 (0.76–1.51)</td>
</tr>
<tr>
<td>c.35G&gt;C (p.G12A)</td>
<td>Wild type</td>
<td>19</td>
<td>6</td>
<td>1.36 (0.60–3.08)</td>
<td>0.56 (0.24–1.30)</td>
</tr>
<tr>
<td>c.35G&gt;T (p.G12V)</td>
<td>Wild type</td>
<td>93</td>
<td>37</td>
<td>1.84 (1.28–2.64)</td>
<td>2.00 (1.38–2.90)</td>
</tr>
<tr>
<td>c.38G&gt;A (p.G13D)</td>
<td>Wild type</td>
<td>103</td>
<td>31</td>
<td>1.33 (0.90–1.96)</td>
<td>0.88 (0.59–1.30)</td>
</tr>
</tbody>
</table>

NOTE: The multivariate Cox regression model included the same set of covariates selected as in Table 3. A P value for significance was adjusted for multiple hypothesis testing to P = 0.05/7 = 0.007. Thus, a P value between 0.05 and 0.007 should be regarded as of borderline significance.

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, 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. Subsequently, 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). Guerrero 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.34G>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 treatment. 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 chemotherapeutic 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 database, we assessed the effects of KRAS codon 12 and 13 mutations independent of various clinico-pathologic 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.

References

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

Yu Imamura, Teppei Morikawa, Xiaoyun Liao, et al.

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