Prognostic Role of PIK3CA Mutation in Colorectal Cancer: Cohort Study and Literature Review

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Abstract

Purpose: Mutations in PIK3CA [the gene encoding the p110α catalytic subunit of phosphatidylinositol-3-kinase (PI3K)] play an important role in colorectal carcinogenesis. Experimental evidence suggests that PIK3CA exon 9 and exon 20 mutations trigger different biologic effects, and that concomitant mutations in both exons 9 and 20 synergistically enhance tumorigenic effects. Thus, we hypothesized that PIK3CA exon 9 and exon 20 mutations might have differential effects on clinical outcome in colorectal cancer, and that concomitant PIK3CA exon 9 and 20 mutations might confer aggressive tumor behavior.

Experimental Design: We sequenced PIK3CA by pyrosequencing in 1,170 rectal and colon cancers in two prospective cohort studies, and found 189 (16%) PIK3CA mutated tumors. Mortality HR according to PIK3CA status was computed using Cox proportional hazards model, adjusting for clinical and molecular features, including microsatellite instability, CpG island methylator phenotype, LINE-1 methylation, and BRAF and KRAS mutations.

Results: Compared with PIK3CA wild-type cases, patients with concomitant PIK3CA mutations in exons 9 and 20 experienced significantly worse cancer-specific survival [log-rank P = 0.031; multivariate HR = 3.51; 95% confidence interval (CI): 1.28–9.62] and overall survival (log-rank P = 0.0086; multivariate HR = 2.68; 95% CI: 1.24–5.77). PIK3CA mutation in either exon 9 or 20 alone was not significantly associated with patient survival. No significant interaction of PIK3CA mutation with BRAF or KRAS mutation was observed in survival analysis.

Conclusion: Coexistence of PIK3CA (the PI3K p110α subunit) exon 9 and 20 mutations, but not PIK3CA mutation in either exon 9 or 20 alone, is associated with poor prognosis of colorectal cancer patients. Clin Cancer Res; 18(8); 2257–68. ©2012 AACR.

Introduction

Phosphatidylinositol-3-kinases (PI3K) are lipid kinases that promote various biologic processes, including cellular proliferation and survival (1). Mutations in the PIK3CA gene, which encodes the p110α catalytic subunit of PI3K, have been identified in many human solid tumors, including colon, breast, brain, ovarian, liver, and lung cancers (1). In colorectal cancers, PIK3CA mutations, which are found in 10% to 20% of tumors, have been reported to be associated with specific clinicopathologic features and molecular events, such as proximal tumor location, microsatellite instability (MSI), and KRAS mutation (2–10).

The prognostic significance of PIK3CA mutation in colorectal cancer remains unclear (Table 1; refs. 2, 4, 7, 8, 11–16). The majority of activating PIK3CA mutations map to 3 sites: exon 9, codons 542 and 545 in the helical domain, and exon 20, codon 1,047 in the kinase domain. Mutation at any one of these sites has been shown to result in a gain of enzymatic function and to promote oncogenic transformation in vitro and in vivo (17–19). Interestingly, the mechanisms through which helical and kinase domain mutations augment enzyme function differ (20). Furthermore, the coexistence of mutations in both exons 9 and 20 of the same p110α molecule (PIK3CA) leads to a synergistic gain of function, with a potent transforming capacity in vitro (20). Thus, we hypothesized that PIK3CA exon 9 and exon...
20 mutations might have differential effects on tumor behavior, and that the coexistence of mutations in both exons 9 and 20 might result in more aggressive tumor behavior compared with cancers with wild-type PIK3CA, or a single mutation in either exon 9 or exon 20.

The interaction of epidermal growth factor with epidermal growth factor receptor triggers 2 main signaling pathways, RAS–RAF–MAPK and PI3K–Akt. Activation of these pathways by mutations in KRAS, BRAF, and/or PIK3CA is an established mechanism that drives colorectal carcinogenesis (21). In thyroid cancers, the coexistence of BRAF and PIK3CA mutations is associated with aggressive tumor behavior (22, 23). On the basis of these findings, our third hypothesis was that PIK3CA and BRAF mutations might interact synergistically to confer a more aggressive colorectal cancer phenotype.

To test these hypotheses, we used our molecular pathology epidemiology (24–26) database based on 2 ongoing U.S. nationwide prospective cohort studies. We assessed various additional molecular features, including KRAS mutation, CpG island methylator phenotype (CIMP), MSI, TP53 negativity, and LINE-1 hypomethylation, and could therefore control for confounding by these potential predictors of outcome.

Materials and Methods

Study group

We used the database of 2 prospective cohort studies, the Nurses’ Health Study (NHS, N = 121,700 women observed since 1976) and the Health Professionals Follow-Up Study (HPFS, N = 51,500 men observed since 1986). Paraffin-embedded tissue blocks were collected from hospitals with participants with colorectal cancer who underwent resection of their primary tumors. We collected diagnostic biopsy specimens for rectal cancers patients who received preoperative therapy to avoid treatment-related artifact or bias. The tissue retrieval rate was approximately 70% when specimens were requested within 5 years of diagnosis. All colorectal cancer cases were confirmed through review of histology by a pathologist (S.O.) blinded to other data. Tumor grade was categorized as high (≤50% glandular area) or low (>50% glandular area). On the basis of the availability of DNA (at least some amount of DNA was available in 1,267 cases), PIK3CA sequencing data, and survival data, a total of 1,170 colorectal cancer cases diagnosed up to 2006 were included in this study. Patients were observed until death, or January 2011, whichever came first. Ascertainment of deaths included reporting by family members or, where study correspondence had been returned, by postal authorities. The National Death Index was used to ensure completeness of ascertainment. The cause of death was assigned by study physicians. Written informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women’s Hospital.

Translational Relevance

PIK3CA mutation is present in various human cancers and plays a role in cancer cell proliferation and survival. The relationship between PIK3CA mutation in colorectal cancer and patient survival remains controversial. In this study we used a database of 1,170 colorectal cancers in 2 prospective cohort studies. Our study benefited from adequate participant follow-up and the availability of clinical information and data on additional molecular characteristics that are important in colorectal carcinogenesis. This is, by far, the largest study on the prognostic role of PIK3CA mutations in colorectal cancer to date, and it suggests that patients with concomitant PIK3CA mutations in both exons 9 and 20 might be associated with worse survival. The presence of a single PIK3CA mutation in either exon 9 or 20 was not significantly associated with patient survival. Considering the role of the phosphatidylinositide-3-kinase signaling pathway in cancer, our findings might be relevant toward personalized medicine.

Sequencing of PIK3CA, BRAF and KRAS, and MSI analysis

Genomic DNA was extracted from paraffin-embedded tissue. Methods for PCR and pyrosequencing targeted at PIK3CA exons 9 and 20 were adapted from those previously described (10) with the following modifications: we replaced the sequencing primer PIK3CA 9-RS2 with 5′-TTTCCTCCT/GCTT/CATGATTTT-3′ and employed a new nucleotide dispensation order (ATAGACGTGCAGCTAGCTAGCTAGCTAGCT), which was particularly sensitive for c.1624G > A; sequencing primer PIK3CA 9-RS3 was replaced with 5′-TAGAAAAATTTTTTCTTCTGTG-3′, and a new dispensation order (ATAGACGTGCAGCTAGCTAGCTAG) used to detect the most common mutations, c.1633G > A and c.1624G > A.

PCR and pyrosequencing assays targeted at BRAF (codon 600; ref. 27) and KRAS (codons 12 and 13) mutations (28) were carried out as previously described. MSI was assessed using a panel of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, D18S55, D18S56, D18S67, and D18S487; ref. 29). MSI high was defined as the presence of instability in 30% or more of the markers and MSI low/microsatellite stability (MSS) as 0% to 29% unstable markers (29).

Analysis of CpG island methylation and LINE-1 hypomethylation

Sodium bisulfite treatment of DNA and real-time PCR assays (MethyLight) were carried out as previously described (29, 30). We quantified promoter methylation at 8 CIMP-specific loci: CACNA1G, CDKN2A (p16), CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1 (31–33). CIMP high was defined as 6 or more (of 8) methylated promoters, and CIMP low/0 as 0 to 5 (of 8)
methylation changes on a background of relatively high methylation, a LINE-1 PCR pyrosequencing assay was employed (34, 35).

TP53 immunohistochemistry

Tissue microarray blocks were constructed and immunohistochemistry for TP53 (p53) was carried out (36). Positive and negative controls were included in each run of immunohistochemistry. All immunostaining slides were scored by a pathologist (S.O.) blinded to other data. A random sample of 118 tumors was reexamined by a second observer (K.N.) unaware of other data. The concordance between the 2 observers was 0.87 (κ = 0.75; P < 0.0001), indicating substantial agreement.

Statistical analysis

All statistical analyses were done using SAS software (version 9.1; SAS Institute Inc.). For categorical data, the χ² test or Fisher exact test was carried out. All P values were 2-sided. When multiple hypothesis testing was carried out, the P value for significance was adjusted to P = 0.0038 (= 0.05/13) by Bonferroni correction. To compare mean age and mean LINE-1 methylation levels, a t test or ANOVA, assuming equal variances, was carried out. To assess whether associations between PIK3CA mutation and the variables in Table 2 were independent of other variables, a multivariate logistic regression analysis was conducted for cross-sectional analyses. ORs were adjusted for age at diagnosis (continuous), sex, tumor location (proximal vs. distal), CIMP status (high vs. low/0), MSI status (high vs. low/MSS), LINE-1 methylation (continuous), BRAF mutation, and KRAS mutation. A backward stepwise elimination with a threshold of P = 0.05 was used to select variables in the final model to avoid overfitting.

The Kaplan–Meier method and the log-rank test were done for survival analysis. Deaths from causes other than colorectal cancer were censored in colorectal cancer–specific mortality analyses. We carried out power calculations. Assuming a total number of patients of 1,170, 7 cases with PIK3CA mutations in both exons 9 and 20, 50% mortality, and an alpha (type I error rate) of 0.05, there was a 50% power to detect an HR of 4.6. To control for confounding, we used Cox proportional hazards models to calculate HR of death according to tumor PIK3CA status, adjusting for age at diagnosis (continuous), sex (NHIS vs. HPFS), year of diagnosis (continuous), tumor location (proximal vs. distal colon vs. rectum), tumor grade, MSI (high vs. low/MSS), CIMP (high vs. low/0), LINE-1 methylation (continuous), KRAS mutation, and BRAF mutation. To minimize residual confounding and overfitting, disease stages I, II, III, IV, or unknown) were used as a stratifying variable using the “strata” option in the SAS proc phreg command. To avoid overfitting, variables in the final model were selected using backward stepwise elimination with a threshold of P = 0.05. Interaction was assessed using the Wald test on the cross-product of PIK3CA and another variable of interest (excluding cases with missing data) in a multivariate Cox model.

The proportionality of hazards assumption was satisfied by evaluating time-dependent variables, which were the cross-products of the PIK3CA variable and survival time (P = 0.44 for colon cancer–specific mortality; P = 0.95 for overall mortality).

To avoid overfitting, cases with missing data in any of the categorical variables [tumor location (0.6%), tumor grade (0.6%), CIMP (6.6%), MSI (1.4%), BRAF (0.5%), and KRAS (0.4%)] were included in the majority category for that variable. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter the results (data not shown).

Results

PIK3CA mutation status in colorectal cancer

PIK3CA pyrosequencing analysis was successful in 95.6% (1,212/1,267) of colorectal cancers. Pyrosequencing has been shown to be a reproducible, precise, and sensitive method of mutation analysis in paraffin-embedded tumor tissues (10, 28). Cases lacking survival data were excluded. Of the remaining 1,170 colorectal cancer cases, 536 cases were from HPFS (men) and 634 cases were from NHS (women).

PIK3CA mutation was detected in 189 (16%) of 1,170 cases, among which 109 cases (58%) had mutations in only exon 9, 73 cases (39%) had mutations in only exon 20, and 7 cases (3.7%) had mutations in both exons 9 and 20. Supplementary Table S1 shows the frequencies of specific PIK3CA mutations. The relationships between PIK3CA mutation and clinical, pathologic, and molecular features in each cohort (NHIS and HPFS) are presented separately in Supplementary Table S2 and Supplementary Table S3, and show general consistency between the 2 cohorts. Table 2 summarizes clinical, pathologic, and molecular features of colorectal cancer according to PIK3CA mutation status.

In contrast to mutations in exon 9, mutations in exon 20 were associated with MSI high (P = 0.0007), CIMP high (P = 0.028), and BRAF mutation (P = 0.030). Associations between concomitant PIK3CA exon 9 and exon 20 mutation status and family history of colorectal cancer (P = 0.030), MSI high (P = 0.0050), CIMP high (P = 0.025), and TP53 negativity (P = 0.0080) were of borderline significance taking into account multiple hypothesis testing (requiring P = 0.0038 as a significance level). Supplementary Table S4 shows the clinical, pathologic, and molecular features of colorectal cancers categorized by overall PIK3CA mutation status (any mutation vs. no mutation). PIK3CA overall mutation status was significantly associated with KRAS mutation (P < 0.0001); however, there was no significant difference in the frequency of KRAS mutation between exon 9 and exon 20 mutants (P = 0.29).

Detailed information on the 7 cases with concomitant PIK3CA exon 9 and exon 20 mutations is shown in Table 3. One case had 3 PIK3CA mutations (c.1624G>A, c.1631C>A, and c.3140A>G). Although the number of patients with cancers harboring concomitant PIK3CA exon 9 and exon 20 mutations (3/1,170 cases; 0.3%) is small, it is noteworthy that this PIK3CA mutation pattern has been associated with poor outcomes in breast cancers (37).
<table>
<thead>
<tr>
<th>Authors (Ref.)</th>
<th>No. of hospitals</th>
<th>Total no. events*</th>
<th>Sample size</th>
<th>Tumor location</th>
<th>Disease stage</th>
<th>No. of PIK3CA mutants</th>
<th>BRAF data</th>
<th>KRAS data</th>
<th>CS, OS, DFS, RFS or PFS log-rank P</th>
<th>Multivariate HR (95% CI), P</th>
<th>Notes and/or a list of variables examined in multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kato and colleagues (2)</td>
<td>1</td>
<td>32</td>
<td>158</td>
<td>Colon &amp; rectum</td>
<td>III/IV</td>
<td>11</td>
<td>No Yes</td>
<td>0.022 (RFS)</td>
<td>2.48 (1.03–5.97) P = 0.043 (RFS)</td>
<td></td>
<td>Lymph node metastasis, CEA level, tumor size and lymphatic invasion.</td>
</tr>
<tr>
<td>Abubaker and colleagues (4)</td>
<td>1</td>
<td>N/A</td>
<td>418</td>
<td>Colon &amp; rectum</td>
<td>I–IV</td>
<td>38</td>
<td>No No</td>
<td>0.036 (CS) NS (OS)</td>
<td>Exon 9 or 20</td>
<td>Exon 9 or 20</td>
<td></td>
</tr>
<tr>
<td>Barault and colleagues (7)</td>
<td>3</td>
<td>197</td>
<td>586</td>
<td>Colon</td>
<td>I–IV</td>
<td>46</td>
<td>Yes Yes</td>
<td>N/A</td>
<td>2.1 (1.2–3.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Souglakos and colleagues (8)</td>
<td>2</td>
<td>43</td>
<td>92</td>
<td>Colon &amp; rectum</td>
<td>I–IV</td>
<td>18</td>
<td>Yes Yes</td>
<td>N/A</td>
<td>2.1 (1.2–3.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarbore-Bianchi and colleagues (11)</td>
<td>2</td>
<td>88</td>
<td>110</td>
<td>Colon &amp; rectum</td>
<td>III–IV</td>
<td>4</td>
<td>No Yes</td>
<td>0.0035 (PFS)</td>
<td>Exon 9 or 20</td>
<td>Exon 9 or 20</td>
<td></td>
</tr>
<tr>
<td>Ogino and colleagues (12)</td>
<td>Many</td>
<td>152</td>
<td>450</td>
<td>Colon</td>
<td>I–III</td>
<td>82</td>
<td>Yes Yes</td>
<td>0.075 (CS)</td>
<td>2.23 (1.21–4.11; CS) Exon 9 or 20</td>
<td></td>
<td>Age, sex, body mass index, year of diagnosis, tumor location, stage, grade and status of MSI, CIMP, KRAS, BRAF, LINE-1 methylation and TP53.</td>
</tr>
<tr>
<td>He and colleagues (13)</td>
<td>Many</td>
<td>84</td>
<td>240</td>
<td>Rectum</td>
<td>I–III</td>
<td>12</td>
<td>Yes Yes</td>
<td>0.008 (LR) NS (OS)</td>
<td>Exon 9 or 20</td>
<td>Exon 9 or 20</td>
<td></td>
</tr>
<tr>
<td>De Roock and colleagues (14)</td>
<td>11</td>
<td>N/A</td>
<td>743</td>
<td>Colon &amp; rectum</td>
<td>IV</td>
<td>74</td>
<td>No No</td>
<td>0.013 (PFS)</td>
<td>2.27 (1.10–4.66) P = 0.042 (PFS)</td>
<td>3.30 (1.46–7.45) P = 0.012 (OS) Exon 20</td>
<td>Age, sex, number of previous chemotherapy lines, center, mutation status of KRAS, BRAF and NRAS. Exon 9 mutation was not associated with outcome.</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Table 1. Studies on prognostic significance of **PIK3CA** exon 9 and 20 mutations in colorectal cancer (Cont’d)

<table>
<thead>
<tr>
<th>Authors (Ref.)</th>
<th>No. of hospitals</th>
<th>Total no. of events*</th>
<th>Sample size</th>
<th>Tumor location</th>
<th>Disease stage</th>
<th>No. of <strong>PIK3CA</strong> mutants</th>
<th>No. of <strong>BRAF</strong> data</th>
<th>No. of <strong>KRAS</strong> data</th>
<th>CS, OS, DFS, RFS or PFS log-rank P</th>
<th>Multivariate HR (95% CI), P</th>
<th>Notes and/or a list of variables examined in multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tol and colleagues (15)</td>
<td>Many</td>
<td>N/A</td>
<td>436</td>
<td>Colon &amp; rectum</td>
<td>IV</td>
<td>32</td>
<td>11</td>
<td>Yes</td>
<td>Yes</td>
<td>NS (PFS, OS)</td>
<td>Exon 9 or 20 Serum LDH, number of affected organs and previous adjuvant therapy. Exon 9 mutation was not associated with outcome.</td>
</tr>
<tr>
<td>Farina Sarasqueta and colleagues (16)</td>
<td>?</td>
<td>?</td>
<td>685</td>
<td>Colon</td>
<td>I–III</td>
<td>66</td>
<td>17</td>
<td>No</td>
<td>No</td>
<td>Exon 20</td>
<td>Exon 20 Exon 9 mutation was not associated with outcome.</td>
</tr>
<tr>
<td>Liao and colleagues (current study)</td>
<td>Many</td>
<td>552</td>
<td>1,170</td>
<td>Colon &amp; rectum</td>
<td>I–IV</td>
<td>116</td>
<td>80</td>
<td>Yes</td>
<td>Yes</td>
<td>P = 0.04 (DFS), P = 0.03 (CS)</td>
<td>Age, sex, year of diagnosis, tumor location, stage, grade and status of MSI, CIMP, <strong>KRAS</strong>, <strong>BRAF</strong> and LINE-1 methylation.</td>
</tr>
</tbody>
</table>

Abbreviations: CS, cancer-specific survival; DFS, disease-free survival; LR, local recurrence; N/A, not available; NS, not significant; OS, overall survival; PFS, progression-free survival; RFS, relapse-free survival.

* indicates relapses or deaths.
Table 2. Clinical, pathologic, and molecular features of colorectal cancer according to PIK3CA mutation status

<table>
<thead>
<tr>
<th>Feature</th>
<th>Total No. (%)</th>
<th>PIK3CA wild type No. (%)</th>
<th>Only in exon 9 No. (%)</th>
<th>Only in exon 20 No. (%)</th>
<th>In both exon 9 and exon 20 No. (%)</th>
<th>P (across all categories)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no.</td>
<td>1,170</td>
<td>981</td>
<td>109</td>
<td>73</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, (HPFS)</td>
<td>536 (46)</td>
<td>439 (45)</td>
<td>56 (51)</td>
<td>36 (49)</td>
<td>5 (71)</td>
<td>0.26</td>
</tr>
<tr>
<td>Female, (NHS)</td>
<td>634 (54)</td>
<td>542 (55)</td>
<td>53 (49)</td>
<td>37 (51)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Mean age at diagnosis (y) ± SD</td>
<td>68.7 ± 8.7</td>
<td>68.6 ± 8.7</td>
<td>69.4 ± 8.9</td>
<td>0.94</td>
<td>68.3 ± 9.0</td>
<td>75.6 ± 10.0</td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before 1997</td>
<td>501 (43)</td>
<td>424 (43)</td>
<td>43 (39)</td>
<td>31 (43)</td>
<td>3 (43)</td>
<td>0.76</td>
</tr>
<tr>
<td>1997 or after</td>
<td>669 (57)</td>
<td>557 (57)</td>
<td>66 (61)</td>
<td>42 (57)</td>
<td>4 (57)</td>
<td>0.92</td>
</tr>
<tr>
<td>Family history of colorectal cancer in first degree relatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>951 (81)</td>
<td>804 (82)</td>
<td>90 (83)</td>
<td>54 (74)</td>
<td>3 (43)</td>
<td>0.19</td>
</tr>
<tr>
<td>Present</td>
<td>219 (19)</td>
<td>177 (18)</td>
<td>19 (17)</td>
<td>19 (26)</td>
<td>4 (57)</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>258 (22)</td>
<td>230 (24)</td>
<td>17 (16)</td>
<td>10 (14)</td>
<td>1 (14)</td>
<td>0.80</td>
</tr>
<tr>
<td>Distal colon</td>
<td>359 (31)</td>
<td>300 (31)</td>
<td>32 (29)</td>
<td>25 (34)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>546 (47)</td>
<td>444 (45)</td>
<td>60 (55)</td>
<td>38 (52)</td>
<td>4 (57)</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>282 (24)</td>
<td>231 (24)</td>
<td>33 (30)</td>
<td>15 (21)</td>
<td>3 (43)</td>
<td>0.093</td>
</tr>
<tr>
<td>II</td>
<td>327 (28)</td>
<td>270 (28)</td>
<td>28 (26)</td>
<td>28 (38)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>308 (26)</td>
<td>264 (27)</td>
<td>24 (22)</td>
<td>19 (26)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>151 (13)</td>
<td>124 (13)</td>
<td>15 (14)</td>
<td>10 (14)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>102 (9)</td>
<td>92 (9)</td>
<td>9 (8)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1,052 (91)</td>
<td>880 (90)</td>
<td>102 (94)</td>
<td>63 (86)</td>
<td>7 (100)</td>
<td>0.067</td>
</tr>
<tr>
<td>High</td>
<td>111 (9)</td>
<td>95 (10)</td>
<td>6 (6)</td>
<td>10 (14)</td>
<td>0 (0)</td>
<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>978 (85)</td>
<td>820 (85)</td>
<td>100 (92)</td>
<td>52 (72)</td>
<td>6 (86)</td>
<td>0.0007</td>
</tr>
<tr>
<td>MSI high</td>
<td>176 (15)</td>
<td>146 (15)</td>
<td>9 (8)</td>
<td>20 (28)</td>
<td>1 (14)</td>
<td>0.0050</td>
</tr>
<tr>
<td>CIMP status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP low/0</td>
<td>906 (83)</td>
<td>762 (83)</td>
<td>88 (87)</td>
<td>52 (73)</td>
<td>4 (57)</td>
<td>0.028</td>
</tr>
<tr>
<td>CIMP high</td>
<td>187 (17)</td>
<td>152 (17)</td>
<td>13 (13)</td>
<td>19 (27)</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>BRAF status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>993 (85)</td>
<td>831 (85)</td>
<td>99 (91)</td>
<td>57 (79)</td>
<td>6 (86)</td>
<td>0.030</td>
</tr>
<tr>
<td>Mutant</td>
<td>171 (15)</td>
<td>145 (15)</td>
<td>10 (9)</td>
<td>15 (21)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>KRAS status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>747 (64)</td>
<td>659 (67)</td>
<td>48 (44)</td>
<td>38 (53)</td>
<td>2 (29)</td>
<td>0.29</td>
</tr>
<tr>
<td>Mutant</td>
<td>418 (36)</td>
<td>318 (33)</td>
<td>61 (56)</td>
<td>34 (47)</td>
<td>5 (71)</td>
<td></td>
</tr>
<tr>
<td>Mean LINE-1 methylation level (%) ± SD</td>
<td>62.8 ± 9.5</td>
<td>62.5 ± 9.6</td>
<td>64.3 ± 9.6</td>
<td>0.53</td>
<td>63.9 ± 8.9</td>
<td>62.3 ± 7.5</td>
</tr>
<tr>
<td>TP53 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>520 (57)</td>
<td>422 (55)</td>
<td>56 (72)</td>
<td>39 (68)</td>
<td>3 (60)</td>
<td>0.71</td>
</tr>
<tr>
<td>Positive</td>
<td>385 (43)</td>
<td>343 (44)</td>
<td>22 (28)</td>
<td>18 (32)</td>
<td>2 (40)</td>
<td>0.0080</td>
</tr>
</tbody>
</table>

NOTE: The % number indicates the proportion of patients with a specific clinical, pathologic or molecular feature among all patients, or patients with specific PIK3CA mutation status.
mutations was small, this subgroup seemed to have an association with family history of colorectal cancer. Notably, all 7 patients died of colorectal cancer or other causes within the follow-up period.

**Independent association between PIK3CA and KRAS mutations**

We carried out multivariate logistic regression analysis to assess for independent relationships between KRAS mutation, other factors, and PIK3CA overall mutation status. In multivariate model analysis, KRAS mutation remained significantly associated with PIK3CA overall mutation [multivariate OR = 2.65; 95% confidence interval (CI), 1.89–3.73; \( p < 0.0001 \)]. In addition, CIMP status remained in the final model (multivariate OR = 1.65; 95% CI: 1.07–2.54; \( p = 0.024 \)), with borderline significance given multiple hypothesis testing (Supplementary Table S5).

**PIK3CA mutation in colorectal cancer and patient survival**

We assessed the prognostic role of PIK3CA mutation in 1,170 colorectal cancers to test the hypotheses that PIK3CA exon 9 and exon 20 mutations might have differential effects on tumor behavior, and that the presence of mutations in both exon 9 and exon 20 might result in more aggressive tumor behavior. During a median follow-up period of 141 months for survivors (interquartile range: 105–192), there were 552 deaths, including 328 colorectal cancer–specific deaths. Notably, patients with PIK3CA mutations in both exons 9 and 20 (henceforth referred to as ‘exon 9 and 20 double mutants’) experienced significantly shorter cancer-specific survival (log-rank \( p = 0.031 \); Fig. 1A) and overall survival than patients with wild-type PIK3CA (log-rank \( p = 0.0008 \); Fig. 1B). In Cox regression analysis, compared with PIK3CA wild-type cases, exon 9 and 20 double mutant status was associated with significantly higher colorectal cancer–specific mortality (univariate HR = 2.84; 95% CI: 1.05–7.69; multivariate HR = 3.51; 95% CI: 1.28–9.62) and overall mortality (univariate HR = 3.37; 95% CI: 1.58–7.15; multivariate HR = 2.84; 95% CI: 1.05–7.69; multivariate HR

\[ \text{HR}_{\text{multivariate}} = 3.37; \ 95\% \ CI: 1.58–7.15; \text{HR}_{\text{multivariate}} \text{CI} = 1.05–7.69; \]

When each cohort was analyzed separately, overall PIK3CA mutation status was not significantly associated with colorectal cancer–specific or overall survival (Supplementary Table S7). HRs were similar for both cohorts and 95% CI were largely overlapping, showing the consistency of results between the 2 cohorts.

**Combined PIK3CA and BRAF, KRAS mutation status, and colorectal cancer prognosis**

To test the third hypothesis that the presence of both BRAF and PIK3CA mutations might result in aggressive tumor behavior, we examined combined BRAF and PIK3CA mutation status and patient prognosis (Supplementary Table S8). Compared with PIK3CA wild-type/BRAF wild-type cases, the presence of mutations in both PIK3CA and BRAF was not significantly associated with colorectal cancer–specific mortality in univariate analysis (HR = 1.24; 95% CI: 0.61–2.52). However, in multivariate analysis, the presence of mutations in both PIK3CA and BRAF was significantly associated with colorectal cancer–specific mortality (multivariate HR = 2.40; 95% CI: 1.12–5.16). We found that MSI and CIMP status were confounders; when we simply adjusted for MSI and CIMP, the adjusted HR (PIK3CA mutated/BRAF mutated vs. PIK3CA wild-type/BRAF wild-type) was 3.08 (95% CI: 1.44–6.61).

We also examined the influence of KRAS and BRAF mutation status on the prognostic association of mutations in PIK3CA. We classified colorectal cancers into 3 subtypes according to KRAS and BRAF status: BRAF wild type/KRAS wild type, BRAF mutated/KRAS wild type, and BRAF wild type/KRAS mutated. No substantial effect modification by KRAS/BRAF mutation status was observed in survival analyses (Table 5).

**PIK3CA mutation status and mortality in strata of other variables**

In further exploratory analyses, the prognostic effect of PIK3CA mutation in strata of other variables was evaluated. The effect of PIK3CA on cancer-specific mortality did not significantly differ according to disease stage (\( P_{\text{interaction}} = 0.93 \)), tumor location (\( P_{\text{interaction}} = 0.099 \)), or any of the other variables examined (all \( P_{\text{interaction}} > 0.05 \)).

**Discussion**

We conducted this study to test the hypotheses that PIK3CA exon 9 and exon 20 mutations might have differential effects on colorectal cancer behavior, and that the presence of concomitant mutations in both exons 9 and 20 might lead to aggressive tumor behavior. We found no significant association between overall or exon-specific PIK3CA mutation status and survival. The concomitant presence of mutations in both exons 9 and 20 was, however, associated with a poorer prognosis for colorectal cancer patients, although confirmation by other studies would be essential. Our data support the hypothesis that concomitant exon 9 and 20 mutations may have a synergistic effect on tumor behavior, and are consistent with experimental data by Zhao and colleagues (20) that show potent synergistic effect.
transforming effects of concomitant PIK3CA exons 9 and 20 mutations. Patients with concomitant mutations in exons 9 and 20 of PIK3CA were more likely to report a family history of colorectal cancer. At present, the cause of this potential association remains obscure. One could speculate that a family history of colorectal cancer might confer a genetic predisposition to the development of the PIK3CA mutations. The number of cases with the concomitant PIK3CA

Table 3. Clinical, pathologic, and molecular data of colorectal cancers with concomitant PIK3CA mutations in both exons 9 and 20

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Age</th>
<th>Sex</th>
<th>No. of first-degree relatives with colorectal cancer</th>
<th>Tumor location</th>
<th>TNM stage</th>
<th>Size (cm)</th>
<th>Tumor grade</th>
<th>Exon 9 Nucleotide change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83</td>
<td>Male</td>
<td>0</td>
<td>A</td>
<td>T2N0M0</td>
<td>4.2</td>
<td>Low</td>
<td>c.1633G &gt; A</td>
</tr>
<tr>
<td>2</td>
<td>69</td>
<td>Male</td>
<td>1</td>
<td>S</td>
<td>T3N2M1</td>
<td>3.5</td>
<td>High</td>
<td>c.1633G &gt; A</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>Female</td>
<td>1</td>
<td>SF</td>
<td>T3N0M0</td>
<td>4.5</td>
<td>Low</td>
<td>c.1624G &gt; A</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>Female</td>
<td>0</td>
<td>T</td>
<td>T3N2M0</td>
<td>4.5</td>
<td>High</td>
<td>c.1633G &gt; A</td>
</tr>
<tr>
<td>5</td>
<td>82</td>
<td>Male</td>
<td>0</td>
<td>R</td>
<td>T3N1M1</td>
<td>3.0</td>
<td>High</td>
<td>c.1631C &gt; A</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>Male</td>
<td>1</td>
<td>A</td>
<td>T2N0M0</td>
<td>2.0</td>
<td>Low</td>
<td>c.1633G &gt; A</td>
</tr>
<tr>
<td>7</td>
<td>88</td>
<td>Male</td>
<td>1</td>
<td>A</td>
<td>T2N0M0</td>
<td>4.3</td>
<td>Low</td>
<td>c.1633G &gt; A</td>
</tr>
</tbody>
</table>

Abbreviations: A, ascending colon; DFO, died from other causes; DOD, died of disease (colorectal cancer); H, MSI high; NA, not available; R, rectum; S, sigmoid; SF, splenic flexure; T, transverse colon; WT, wild type.

Figure 1. Kaplan–Meier curves for colorectal cancer–specific (A) and overall survival (B) according to PIK3CA exon–specific mutation status in colorectal cancer. Kaplan–Meier curves for colorectal cancer–specific (C) and overall survival (D) according to overall PIK3CA mutation status in colorectal cancer.
In our current study, neither PIK3CA overall mutation status, nor PIK3CA mutation in exon 9 or 20 alone, was significantly associated with patient survival. This is in contrast to some of the published literature. Notwithstanding the potential for confounding by BRAF status in other studies, it is worth bearing in mind that small studies with null results have a higher probability of being unpublished compared to similarly sized datasets with "significant" findings. Large studies with adequate statistical power are less prone to this type of "publication bias." We should therefore place more emphasis on the results of large-scale studies when we evaluate publications on the prognostic significance of cancer biomarkers. Furthermore, experimental evidence suggests that PIK3CA mutation alone has a relatively modest effect on tumor cell growth, and that

Table 4. PIK3CA mutation in colorectal cancer and patient mortality

<table>
<thead>
<tr>
<th>PIK3CA status</th>
<th>Total No.</th>
<th>Univariate HR (95% CI)</th>
<th>Stage-stratified HR (95% CI)</th>
<th>Overall mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype (exon 9 or 20)</td>
<td>981</td>
<td>277</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>Mutant (exon 9 or 20)</td>
<td>189</td>
<td>51</td>
<td>0.95 (0.70–1.28)</td>
<td>1.03 (0.77–1.40)</td>
</tr>
<tr>
<td>Mutant in only exon 9</td>
<td>109</td>
<td>29</td>
<td>0.94 (0.64–1.38)</td>
<td>1.05 (0.72–1.55)</td>
</tr>
<tr>
<td>Mutant in only exon 20</td>
<td>73</td>
<td>18</td>
<td>0.83 (0.52–1.34)</td>
<td>0.87 (0.4–1.45)</td>
</tr>
<tr>
<td>Mutations in both exon 9 and exon 20</td>
<td>7</td>
<td>4</td>
<td>2.84 (1.05–7.69)</td>
<td>3.61 (1.32–9.87)</td>
</tr>
<tr>
<td>NOTE: The multivariate, stage-stratified Cox regression model initially included age, sex, year of diagnosis, tumor location, tumor grade, MSI, CpG island methylator phenotype, KRAS mutation, BRAF mutation and LINE-1 methylation. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Clinical, pathologic, and molecular data of colorectal cancers with concomitant PIK3CA mutations in both exons 9 and 20 (Cont’d)
PIK3CA mutations need to cooperate with other PI3K enzyme mutations for effective cellular transformation (17, 49). Because of the power limitations in the previous studies on PIK3CA mutation, we feel that there is currently insufficient evidence to support a role for PIK3CA exon 9 or 20 mutation alone as a prognostic biomarker in colorectal cancer. Our findings warrant validation in additional large cohort studies.

We previously described an association between PIK3CA mutation and shorter cancer-specific survival among 450 stages I to III colon cancer cases (12). All of those 450 cases were included in our current study. Sample size and adequate statistical power are critically important in such exploratory studies (48). Because our previous study was restricted to stages I to III colon cancers, the numbers of adverse events (66 colon cancer–specific deaths and 152 overall deaths) were also much smaller than in our current study (328 colorectal cancer–specific deaths and 552 overall deaths), which included cancers of all stages. As a result, our current findings are more robust than those of our previous study in which, as a result of smaller sample size and lower power, there would have been increased risk of finding spurious associations. This underscores the critical importance of careful study design, adequate statistical power, and cautious interpretation of data, which are prerequisites for exploratory studies of this nature (50).

Even in our larger dataset of 1,170 colorectal cancers, there were only 7 patients who harbored PIK3CA mutations in both exons 9 and 20. However, given that over 550,000 individuals are diagnosed with colorectal cancer each year in the United States and Europe, we estimate that, in these regions combined, there would be approximately 3,300 colorectal cancer patients every year with PIK3CA mutations in both exons 9 and 20. The incidence of this potentially aggressive type of colorectal cancer may, in fact, be similar to the combined sum of the incidences of Burkitt lymphoma, hairy cell leukemia, ALK-positive large B-cell lymphoma, and angioimmunoblastic T-cell lymphoma in these Western countries. Other cancers with a similar incidence include osteosarcoma, medulloblastoma, gestational choriocarcinoma, and ovarian clear cell carcinoma. Thus, those colorectal cancers with PIK3CA mutations in both exons 9 and 20 may represent as significant a cancer burden as the other cancer types in our society.

Caveats of our current study include the limited data on cancer treatment in the cohorts, which prevented the inclusion of treatment as a variable in our analyses. Nonetheless, it is unlikely that chemotherapy use or regimens differed substantially by PIK3CA mutation status, given that a vast majority of cases were diagnosed before 2006, and PIK3CA mutation data were unavailable to physicians or patients. In addition, our multivariable Cox regression analysis adjusted for disease stage (I, II, III, or IV), on which treatment decision making was mostly based.

Our findings relating to survival in patients whose tumors harbored mutations in both exons 9 and 20 of PIK3CA are novel. However, given that the number of such cases in our study was small and statistical power was consequently limited, these findings warrant validation by independent studies.

Our study gains several strengths through the use of the database of 2 U.S. nationwide prospective cohort studies. Clinicopathologic information, various exposures, and tumor molecular data have been integrated into our molecular pathologic epidemiology (24–26) database. Cohort participants with colorectal cancer sought medical attention and were treated at hospitals throughout the United States. Hence, our sample is more representative of colorectal cancer in the general U.S. population than a convenience sample collected at one or a few hospitals. Moreover, our extensive tumor database enabled us to assess the
prognostic association of PIK3CA mutations independent of other critical molecular events such as BRAF and KRAS mutations, LINE-1 hypomethylation, MSI, and CIMP, which have all been associated with colon cancer outcome (29, 34).

In conclusion, in our study of 1,170 colorectal cancers, concomitant PIK3CA mutation of both exons 9 and 20 was associated with a poorer prognosis, although statistical power was limited because of only 7 cases with the concomitant mutations. In contrast, neither PIK3CA exon 9 nor exon 20 mutation alone seemed to have substantial prognostic influence. The robustness of our findings would be enhanced by replication in other large studies. Our findings might give additional insight into the relevance of the PI3K pathway in colorectal cancer progression and suggest that detailed genotyping of PIK3CA might serve toward personalized medicine.

Disclosure of Potential Conflicts of Interest
The content is solely the responsibility of the authors and does not necessarily represent the official views of NCI or NIH. Funding agencies did not have any role in the design of the study; the collection, analysis, or interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

Authors' Contributions
Conception and design: C.S. Fuchs, S. Ogino.

References

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Liao, T. Morikawa, Y. Imamura, M. Yamauchi, Z.R. Qian, C.S. Fuchs, S. Ogino.
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): X. Liao, T. Morikawa, A. Kuchiba, J.A. Meyerhardt, C.S. Fuchs, S. Ogino.
Writing, review, and/or revision of the manuscript: X. Liao, T. Morikawa, P. Lochhead, J.A. Meyerhardt, C.S. Fuchs, S. Ogino.

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Prognostic Role of PIK3CA Mutation in Colorectal Cancer: Cohort Study and Literature Review

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