Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

Imaging, Diagnosis, Prognosis

Clinical Cancer Research

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 phosphatidylinositide-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 $P = 0.0008$; 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

Phosphatidylinositide-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

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-11-2410

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

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.

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 pathologic 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 where participants with colorectal cancer 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 ($\leq$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.

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′-TTCTCCTT/GCTT/CAGTGATTT-3′ and employed a new nucleotide dispensation order (ATACACATGTCAGTCAGACTAGCTAGCTAGCTAG), which was particularly sensitive for c.1624G > A; sequencing primer *PIK3CA* 9-RS3 was replaced with 5′-TAGAAAATCTTTCTCCTGCT-3′, and a new dispensation order (ATAGCACTGACTGACTGACTACTGACTGACTG) 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, BAT40, 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)

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

methylated promoters (32). To accurately quantify small 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 ($\kappa = 0.75$; $P < 0.0001$), indicating substantial agreement.

Statistical analysis

All statistical analyses were done using SAS software (version 9.1; SAS Institute Inc.). For categorical data, the χ^2 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 (NHS 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 (NHS 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

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

Table 1. Studies on prognostic significance of *PIK3CA* exon 9 and 20 mutations in colorectal cancer

Authors (Ref.)	No. of hospitals	Total no. of events[a]	Sample size	Tumor location	Disease stage	No. of *PIK3CA* mutants		*BRAF* data	*KRAS* data	CS, OS, DFS, RFS or PFS log-rank *P*	Multivariate HR (95% CI), *P*	Notes and/or a list of variables examined in multivariate analysis
						Exon 9	Exon 20					
Kato and colleagues (2)	1	32	158	Colon & rectum	II/III	11	7	No	Yes	$P = 0.022$ (RFS)	2.48 (1.03–5.97) $P = 0.043$ (RFS)	Lymph node metastasis, CEA level, tumor size and lymphatic invasion.
										$P = 0.036$ (CS)	Exon 9 or 20	
Abubaker and colleagues (4)	1	N/A	418	Colon & rectum	I–IV	38	13	No	No	NS (OS)	Exon 9 or 20	
Barault and colleagues (7)	3	197	586	Colon	I–IV	46	29	Yes	Yes	N/A	N/A	*PIK3CA*, *KRAS* and *BRAF* mutations were not evaluated separately.
											Exon 1, 2, 9 or 20	
Souglakos and colleagues (8)	2	43	92	Colon & rectum	I–IV	18	8	Yes	Yes	NS (OS)	2.1 (1.2–3.9)	Age, tumor grade, metastatectomy, tumor location and number of treatment lines (1 vs. >3). Patients with metastatic colorectal cancer after cetuximab treatment.
											$P = 0.01$ (PFS) Exon 9 or 20	
Sartore-Bianchi and colleagues (11)	2	88	110	Colon & rectum	III–IV	4	11	No	Yes	$P = 0.0035$ (PFS)	Exon 9 or 20	
Ogino and colleagues (12)	Many	152	450	Colon	I–III	82		Yes	Yes	$P = 0.075$ (CS)	2.23 (1.21–4.11; CS) Exon 9 or 20	Age, sex, body mass index, year of diagnosis, tumor location, stage, grade and status of MSI, CIMP, *KRAS*, *BRAF*, LINE-1 methylation and TP53.
He and colleagues (13)	Many	84	240	Rectum	I–III	12	7	Yes	Yes	$P = 0.008$ (LR)	3.4 (1.2–9.2)	TNM stage, circumferential margin.
										NS (OS)	$P = 0.017$ (LR) Exon 9 or 20	
De Roock and colleagues (14)	11	N/A	743	Colon & rectum	IV	74	22	Yes	Yes	$P = 0.013$ (PFS)	2.27 (1.10–4.66) $P = 0.042$ (PFS)	Age, sex, number of previous chemotherapy lines, center, mutation status of *KRAS*, *BRAF* and *NRAS*.
										$P = 0.0057$ (OS)	3.30 (1.46–7.45) $P = 0.012$ (OS) Exon 20	Exon 9 mutation was not associated with outcome.

(Continued on the following page)

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

Table 1. Studies on prognostic significance of *PIK3CA* exon 9 and 20 mutations in colorectal cancer (Cont'd)

Authors (Ref.)	No. of hospitals	Total no. of events[a]	Sample size	Tumor location	Disease stage	No. of *PIK3CA* mutants		*BRAF* data	*KRAS* data	CS, OS, DFS, RFS or PFS log-rank *P*	Multivariate HR (95% CI), *P*	Notes and/or a list of variables examined in multivariate analysis
						Exon 9	Exon 20					
Tol and colleagues (15)	Many	N/A	436	Colon & rectum	IV	32	11	Yes	Yes	NS (PFS, OS)	Exon 9 or 20	Serum LDH, number of affected organs and previous adjuvant therapy.
Farina Sarasqueta and colleagues (16)	?	?	685	Colon	I–III	66	17	No	No	$P = 0.04$ (DFS) $P = 0.03$ (CS)	Exon 20	Exon 9 mutation was not associated with outcome.
Liao and colleagues (current study)	Many	552	1,170	Colon & rectum	I–IV	116	80	Yes	Yes	$P = 0.031$ (CS)	3.51 (1.28–9.62; CS)	Age, sex, year of diagnosis, tumor location, stage, grade and status of MSI, CIMP, *KRAS*, *BRAF* and LINE-1 methylation.
										$P = 0.0008$ (OS)	2.68 (1.24—5.77; OS) Cases with mutations in both exons 9 and 20 1.05 (0.71–1.56; CS) 0.90 (0.67–1.22; OS) Cases with exon 9 mutation alone 0.96 (0.59–1.55; CS) 0.80 (0.55–1.16; OS) Cases with exon 20 mutation alone 1.07 (0.79–1.45; CS) 0.91 (0.72–1.15; OS) Cases with mutations in either or both of exons 9 and 20	

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.

[a], relapses or deaths.

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

			PIK3CA mutation present				
Feature	Total No. (%)	*PIK3CA* wild type No. (%)	Only in exon 9 No. (%)	*P* (exon 9 vs. exon 20)	Only in exon 20 No. (%)	In both exon 9 and exon 20 No. (%)	*P* (across all categories)
Total no.	1,170	981	109		73	7	
Sex				0.88			0.26
Male, (HPFS)	536 (46)	439 (45)	56 (51)		36 (49)	5 (71)	
Female, (NHS)	634 (54)	542 (55)	53 (49)		37 (51)	2 (29)	
Mean age at diagnosis (y) ± SD	68.7 ± 8.7	68.6 ± 8.7	69.4 ± 8.9	0.94	68.3 ± 9.0	75.6 ± 10.0	0.95
Year of diagnosis				0.76			0.92
Before 1997	501 (43)	424 (43)	43 (39)		31 (43)	3 (43)	
1997 or after	669 (57)	557 (57)	66 (61)		42 (57)	4 (57)	
Family history of colorectal cancer in first degree relatives				0.19			0.030
Absent	951 (81)	804 (82)	90 (83)		54 (74)	3 (43)	
Present	219 (19)	177 (18)	19 (17)		19 (26)	4 (57)	
Tumor location				0.80			0.19
Rectum	258 (22)	230 (24)	17 (16)		10 (14)	1 (14)	
Distal colon	359 (31)	300 (31)	32 (29)		25 (34)	2 (29)	
Proximal colon	546 (47)	444 (45)	60 (55)		38 (52)	4 (57)	
Disease stage				0.093			0.23
I	282 (24)	231 (24)	33 (30)		15 (21)	3 (43)	
II	327 (28)	270 (28)	28 (26)		28 (38)	1 (14)	
III	308 (26)	264 (27)	24 (22)		19 (26)	1 (14)	
IV	151 (13)	124 (13)	15 (14)		10 (14)	2 (29)	
Unknown	102 (9)	92 (9)	9 (8)		1 (1)	0 (0)	
Tumor grade				0.067			0.27
Low	1,052 (91)	880 (90)	102 (94)		63 (86)	7 (100)	
High	111 (9)	95 (10)	6 (6)		10 (14)	0 (0)	
MSI status				0.0007			0.0050
MSI low/MSS	978 (85)	820 (85)	100 (92)		52 (72)	6 (86)	
MSI high	176 (15)	146 (15)	9 (8)		20 (28)	1 (14)	
CIMP status				0.028			0.025
CIMP low/0	906 (83)	762 (83)	88 (87)		52 (73)	4 (57)	
CIMP high	187 (17)	152 (17)	13 (13)		19 (27)	3 (43)	
BRAF status				0.030			0.15
Wild type	993 (85)	831 (85)	99 (91)		57 (79)	6 (86)	
Mutant	171 (15)	145 (15)	10 (9)		15 (21)	1 (14)	
KRAS status				0.29			0.0001
Wild type	747 (64)	659 (67)	48 (44)		38 (53)	2 (29)	
Mutant	418 (36)	318 (33)	61 (56)		34 (47)	5 (71)	
Mean LINE-1 methylation level (%) ± SD	62.8 ± 9.5	62.5 ± 9.6	64.3 ± 9.6	0.53	63.9 ± 8.9	62.3 ± 7.5	0.79
TP53 expression				0.71			0.0080
Negative	520 (57)	422 (55)	56 (72)		39 (68)	3 (60)	
Positive	385 (43)	343 (44)	22 (28)		18 (32)	2 (40)	

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.

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

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 = 2.68; 95% CI: 1.24–5.77; Table 4).

In contrast, the presence of a single *PIK3CA* mutation, in either exon 9 or 20, was not significantly associated with patient survival (Fig. 1A and B; Table 4). The prognostic association of *PIK3CA* exon 9 mutations in colorectal cancer (regardless of exon 20 status) was examined. No significant difference was found between exon 9 mutants and wild-type cases in colorectal cancer–specific or overall survival. The HRs for exon 9 mutants were not influenced by the inclusion of double mutants, even after adjusting for exon 20 mutation status. Likewise, the prognostic association of *PIK3CA* exon 20 mutations (regardless of exon 9 status) was similar to wild-type cases in colorectal cancer–specific and overall survival analysis (Supplementary Table S6). Overall *PIK3CA* mutation status (exon 9 or 20 mutation) was not significantly associated with colorectal cancer–specific or overall survival when compared with wild-type cases (Fig. 1C and D; Table 4). 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 ($P_{interaction} = 0.93$), tumor location ($P_{interaction} = 0.099$), or any of the other variables examined (all $P_{interaction} > 0.05$).

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

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

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

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

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.
[a]CIMP status; H, CIMP high; L, CIMP low.

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*

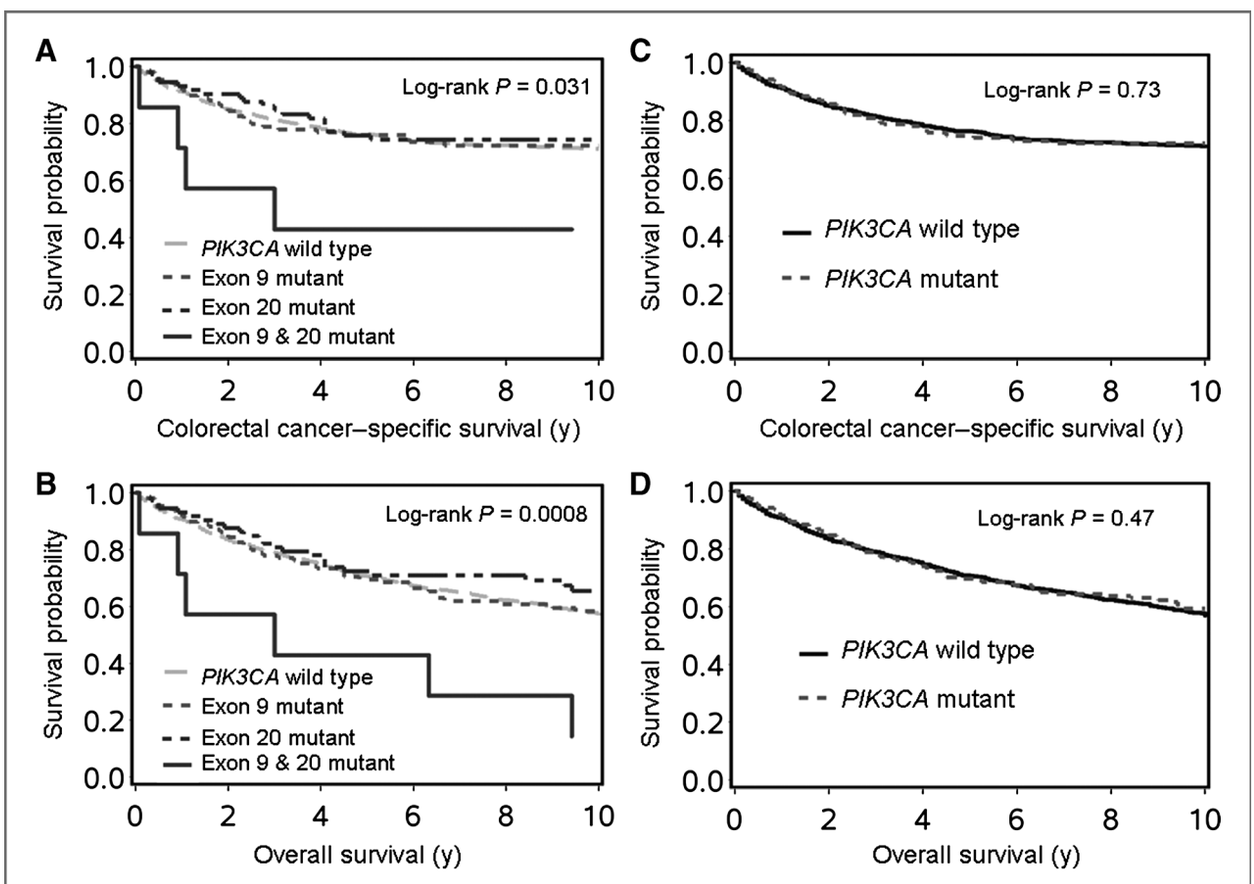

Figure 1. Kaplan–Meier curves for colorectal cancer–specific (A) and overall survival (B) according to *PIK3CA* exon–specific mutation status in colorectal cancer. Kaplan–Meier curves for colorectal cancer–specific (C) and overall survival (D) according to overall *PIK3CA* mutation status in colorectal cancer.

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

Table 3. Clinical, pathologic, and molecular data of colorectal cancers with concomitant *PIK3CA* mutations in both exons 9 and 20 (Cont'd)

Exon 20 Nucleotide change	*KRAS*	*BRAF*	LINE-1 methylation (%)	MSI	CIMP[a]	TP53 expression	Follow-up (mo)	Clinical outcome
c.3140A > T	c.35G > T	WT	59	MSS	L	(–)	162	DFO
c.3129G > T	c.35G > A	WT	63	MSS	L	(+)	11	DOD
c.3137C > A	WT	WT	49	MSS	L	(–)	113	DFO
c.3136G > A	c.35G > A	WT	67	MSS	H	(+)	36	DOD
c.3140A > G c.1631C > A	c.38G > A	WT	59	MSS	L	(–)	13	DOD
c.3140A > G	WT	c.1799T > A	64	H	H	NA	10	DOD
c.3140A > G	c.34G > T	WT	74	MSS	H	NA	76	DFO

exon 9 and 20 mutations was, however, very small, and we should exercise caution in the interpretation of any apparent clinical, pathologic, and molecular associations within this subgroup. These findings must be validated by independent datasets.

The assessment of prognostic factors or biomarkers is important in cancer research (37–45). A number of previous studies have examined the prognostic role of *PIK3CA* mutation in colorectal cancer (Table 1; refs. 2, 4, 7, 8, 11–16, 46). Although some studies have shown that *PIK3CA* mutation is associated with shorter survival (2, 11), the statistical power of these studies is quite limited (sample size $N < 160$). In a larger study ($N = 586$), the presence of a mutation in any of *PIK3CA, BRAF*, or *KRAS* was associated with poor 3-year survival, but the effect of *PIK3CA* mutation in isolation was not studied (7). Two additional studies showed that, compared with patients with wild-type *PIK3CA*, patients with exon 20 mutations experienced worse survival, whereas exon 9 mutation was not associated with outcome (14, 16). In one of these 2 studies, tumor *KRAS* and *BRAF* mutations were not examined (16). In our current study, cancers with *PIK3CA* exon 20 mutation were found to have a higher frequency of *BRAF* mutation. *BRAF* mutation has been associated with a poorer prognosis in colorectal cancer (47) and might therefore confound analyses of *PIK3CA* exon 20 mutation and survival in colorectal cancer. Other groups have reported that *PIK3CA* mutations are not associated with liver metastasis (48) or with overall survival (with or without adjuvant treatment) (4, 8, 15).

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 with 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

		Colorectal cancer–specific mortality				Overall mortality			
PIK3CA status	Total No.	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)
Wildtype	981	277	1 (referent)	1 (referent)	1 (referent)	467	1 (referent)	1 (referent)	1 (referent)
Mutant (exon 9 or 20)	189	51	0.95 (0.70–1.28)	1.03 (0.77–1.40)	1.07 (0.79–1.45)	85	0.92 (0.73–1.16)	0.97 (0.76–1.22)	0.91 (0.72–1.15)
Mutation in only exon 9	109	29	0.94 (0.64–1.38)	1.05 (0.72–1.55)	1.05 (0.71–1.56)	48	0.93 (0.69–1.25)	0.99 (0.73–1.33)	0.90 (0.67–1.22)
Mutation in only exon 20	73	18	0.83 (0.52–1.34)	0.87 (0.4–1.40)	0.96 (0.59–1.55)	30	0.77 (0.53–1.12)	0.80 (0.55–1.15)	0.80 (0.55–1.16)
Mutations in both exon 9 and exon 20	7	4	2.84 (1.05–7.69)	3.61 (1.32–9.87)	3.51 (1.28–9.62)	7	3.37 (1.58–7.15)	3.91 (1.83–8.36)	2.68 (1.24–5.77)

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.

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

Table 5. *PIK3CA* mutation in colorectal cancer and patient mortality according to *KRAS* and *BRAF* mutation status

		Colorectal cancer–specific mortality				Overall mortality			
	Total No.	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)
BRAF wild type and *KRAS* wild type									
PIK3CA (–)	516	124	1 (referent)	1 (referent)	1 (referent)	224	1 (referent)	1 (referent)	1 (referent)
PIK3CA (+)	62	13	0.88 (0.49–1.55)	0.91 (0.51–1.62)	0.97 (0.54–1.74)	23	0.83 (0.54–1.28)	0.88 (0.57–1.36)	0.83 (0.54–1.28)
BRAF mutant and *KRAS* wild type									
PIK3CA (–)	140	41	1 (referent)	1 (referent)	1 (referent)	68	1 (referent)	1 (referent)	1 (referent)
PIK3CA (+)	26	8	1.08 (0.51–2.32)	1.04 (0.48–2.24)	1.67 (0.76–3.69)	12	0.90 (0.49–1.67)	0.88 (0.47–1.64)	1.09 (0.59–2.04)
BRAF wild type and *KRAS* mutant									
PIK3CA (–)	311	109	1 (referent)	1 (referent)	1 (referent)	165	1 (referent)	1 (referent)	1 (referent)
PIK3CA (+)	100	30	0.81 (0.54–1.21)	0.72 (0.48–1.08)	0.69 (0.46–1.04)	50	0.87 (0.64–1.20)	0.82 (0.59–1.13)	0.75 (0.54–1.04)

NOTE: The multivariate, stage-stratified Cox regression model initially included age, sex, year of diagnosis, tumor location, tumor grade, MSI, CpG island methylator phenotype and LINE-1 methylation. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models.

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 medial 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

Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

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.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Liao, T. Morikawa, A. Kuchiba, J.A. Meyerhardt, C.S. Fuchs, S. Ogino.
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Acknowledgments

The authors thank the participants and staff of the Nurses' Health Study and the Health Professionals Follow-Up Study for their valuable contributions as well as the U.S. State Cancer Registries for their help.

Grant Support

This work was supported by U.S. NIH [P01CA87969 (to S.E. Hankinson), P01CA55075 (to W.C. Willett), P50CA127003 (to C.S. Fuchs), and R01CA151993 (to S. Ogino)]; the Bennett Family Fund for Targeted Therapies Research; and the Entertainment Industry Foundation through National Colorectal Cancer Research Alliance.

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Received September 16, 2011; revised January 27, 2012; accepted February 17, 2012; published OnlineFirst February 22, 2012.

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Published OnlineFirst February 22, 2012; DOI: 10.1158/1078-0432.CCR-11-2410

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Clinical Cancer Research

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Clin Cancer Res 2012;18:2257-2268. Published OnlineFirst February 22, 2012.