Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Association Study of the let-7 miRNA-Complementary Site Variant in the 3′ Untranslated Region of the *KRAS* Gene in Stage III Colon Cancer (NCCTG N0147 Clinical Trial)

Dan Sha[1,5], Adam M. Lee[1], Qian Shi[2], Steven R. Alberts[3], Daniel J. Sargent[2], Frank A. Sinicrope[3,4], and Robert B. Diasio[1,3]

Abstract

Purpose: A let-7 microRNA-complementary site (LCS6) polymorphism in the 3′ untranslated region of the *KRAS* gene has been shown to disrupt let-7 binding and upregulate *KRAS* expression. We evaluated the LCS6 genotype and its association with *KRAS* mutation status, clinicopathologic features, and disease-free survival (DFS) in patients with stage III colon cancer who enrolled in a phase III clinical trial (NCCTG N0147).

Experimental Design: The LCS6 genotype was assayed by real-time PCR in DNA extracted from whole blood ($n = 2{,}834$) and compared with paired tumor tissue ($n = 977$). χ^2 and two-sample t tests were used to compare baseline factors and *KRAS* mutation status between patients defined by LCS6 variant status. Log-rank tests and multivariate Cox models assessed associations between LCS6 status and DFS, respectively.

Results: We identified 432 (15.2%) blood samples and 143 (14.6%) tumor samples heterozygous or homozygous for the LCS6 G-allele, and 2,402 of 2,834 (84.8%) blood samples and 834 of 977 (85.4%) tumor samples homozygous for the LCS6 T-allele. Genotype results were highly concordant (99.8%) in cases with paired blood and tumor tissue ($n = 977$). G-allele carriers were significantly more frequent in Caucasians versus other races (χ^2 test, $P < 0.0001$). The LCS6 genotype was not associated with *KRAS* mutation status, clinicopathologic features (all $P > 0.2$), or DFS (log-rank $P = 0.49$; HR, 0.929; 95% confidence interval, 0.76–1.14), even after combining LCS6 genotype with *KRAS* mutation status.

Conclusions: In the largest association study investigating the LCS6 polymorphism in colon cancers, the germline LCS6 genotype was not associated with *KRAS* mutation status or with clinical outcome in patients with stage III tumors. *Clin Cancer Res; 20(12); 3319–27. ©2014 AACR.*

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in men and the second in women throughout the world, with over 1.2 million new cancer cases and 608,700 cancer-related deaths in 2008 (1). In the United States, the estimated new CRC cases and the estimated deaths in 2012 are 143,460 and 51,690, respectively (2). Although tumor stage remains the most important prognostic factor (3, 4), considerable stage-independent variability exists in clinical outcome, which underscores the need for the identification and validation of new predictive and prognostic biomarkers to guide therapeutic decision making for personalized therapy. At present, the only marker that is routinely used in clinical practice is the tumor mutation status of the *KRAS* gene, which predicts nonresponse to anti-EGFR antibodies, including cetuximab, in metastatic patients with CRC (5).

MicroRNAs (miRNA) are endogenous 21- to 22-nucleotide noncoding RNAs (6, 7) that target messenger RNAs (mRNA) and regulate their expression through complementarity to the 3′ UTRs (untranslated region) of mRNAs (8, 9). MiRNAs have been shown to play a role in cancer development and progression (10–13). The lethal-7 (let-7) family is widely viewed as tumor suppressor miRNA, and the expression of let-7 family members is downregulated in cancers of the lung (12), colorectum (14), and breast (15). The human *KRAS* oncogene has been shown to contain multiple let-7 complementary sites (LCS) in its 3′UTR (; ref. 16), which subjects *KRAS* to let-7 miRNA-mediated regulation *in vitro* (14) and in *vivo* (17).

Recent studies have identified a *KRAS* 3′UTR polymorphism (rs61764370), aT-to-G nucleotide change in the sixth LCS (LCS6), that was found to increase *KRAS* expression by altering let-7–binding capability to the *KRAS* mRNA

Authors' Affiliations: [1]Department of Molecular Pharmacology and Experimental Therapeutics (MPET); [2]Alliance Statistics and Data Center; [3]Division of Medical Oncology; [4]Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota; and [5]Department of Medical Oncology, Shandong Provincial Hospital Affiliated to Shandong University, Ji'nan, Shandong Province, China

Dan Sha and Adam Lee contributed equally to this article.

Corresponding Author: Robert B. Diasio, Mayo Clinic Cancer Center, 200 1st St SW, Gonda19-460RES, Rochester, MN 55905. Phone: 507-284-3977; Fax: 507-538-6674; E-mail: Diasio.Robert@mayo.edu

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Translational Relevance

Significant stage-independent variability in clinical outcome of stage III colon cancer continues to be a challenge, highlighting the need for new predictive and prognostic biomarkers. Recent studies have shown potential prognostic value for a *KRAS* 3′ untranslated region polymorphism in the sixth complementary site for the miRNA let-7 (*KRAS*-LCS6); however, the clinical significance of the *KRAS*-LCS6 remains controversial. To determine associations between the *KRAS*-LCS6 variant and patients' *KRAS* mutation status, clinicopathologic features, and disease-free survival, our study used a total of 2,834 patients with stage III colon cancer treated with adjuvant FOLFOX or FOLFIRI, alone or combined with cetuximab. To our knowledge, our study examining the *KRAS*-LCS6 polymorphism is the largest conducted to date. Our results showed no significant association between the *KRAS*-LCS6 variant and clinical outcomes, indicating limited utility as a prognostic marker in stage III colon cancer.

(18). Previous association studies have shown potential prognostic value of the LCS6 variant in early-stage CRC (19) and in patients with metastatic CRC with wild-type (WT) *KRAS* tumors receiving cetuximab (20). However, it's clinical significance and association with *KRAS* mutation status remains controversial due to conflicting results in studies with limited sample sizes (21–23).

Given this prior evidence, we hypothesized that the LCS6 variant is associated with *KRAS* mutation status and may be associated with poor prognosis in colon cancers. We secondarily hypothesized that the LCS6 variant is inversely associated with *BRAF* V600E mutation and deficient DNA mismatch repair (dMMR). To test our hypothesis and further elucidate the significance of the LCS6 variant in a larger patient population, we genotyped the LCS6 variant in a large cohort of patients with stage III colon cancer treated in a randomized trial of FOLFOX alone or combined with cetuximab as postoperative adjuvant chemotherapy (NCCTG N0147). In this study, the addition of cetuximab failed to increase disease-free survival (DFS) compared with FOLFOX alone (24).

Materials and Methods

Study population

Patients were obtained from the NCCTG N0147 Trial, a large randomized phase III study in adjuvant colon cancer designed to assess the potential benefit of cetuximab in resected stage III colon cancer. Patients were enrolled in one of the following treatment arms: FOLFOX ± cetuximab, FOLFIRI ± cetuximab, 6 cycles of FOLFOX followed by 6 cycles of FOLFIRI ± cetuximab, and treatment per local physician discretion. A total of 3,397 patients, of which 2,686 patients with *KRAS* WT, were concurrently randomized to primary comparison arms (FOLFOX + cetuximab vs. FOLFOX). The clinical trial obtained Institutional Review Board approval and all patients provided written informed consent before their participation.

Demographic and clinicopathologic data collection was conducted by the Alliance Statistics and Data Center and included the following: N stage (N1 vs. N2), T stage (T_1/T_2 vs. T_3/T_4), histologic grade [high (poorly differentiated/undifferentiated) vs. low (well/moderately differentiated)], right (proximal) tumor side (cecum, ascending, and transverse colon), or left (distal) tumor side (splenic flexure, descending, and sigmoid colon), and body mass index (BMI; BMI < 20 vs. 20 ≤ BMI < 25 vs. 25 ≤ BMI < 30 vs. BMI ≥ 30). In addition, previously reported data on KRAS (c.35 G>C G12A, c.35 G>A G12D, c.34 G>C G12R, c.34 G>T G12C, c.34 G>A G12S, c.35 G>T G12V, and c.38 G>A G13D) and BRAF (c.1799 T>A V600E) mutations and DNA mismatch repair proteins (dMMR vs. pMMR) were also available (24, 25).

KRAS LCS6 genotyping

A total of 2,834 patients with stage III colon cancer with available DNA from whole blood ($N = 2{,}834$) and paired formalin-fixed paraffin-embedded tumor specimens ($N = 977$) were used for LCS6 genotyping. A previously published probe-based assay (Life Technologies) was used to determine LCS6 variant status (26). PCR primer and probe sequences were as follows: forward primer: GCCAGGCTGGTCTCGAA, reverse primer: CTGAATAAATGAGTTCTGCAAAACAGGTT, reporter sequence 1: CTCAAGTGATTCACCCAC-VIC, and report sequence 2: CAA GTGATGCACCCAC-FAM. Amplification and variant detection was performed using the LightCycler 480 RT-PCR system (Roche Applied Science). To ensure accurate calls, all genotyping plates contained three Coriell DNA samples with known LCS6 variant genotypes (NA12874, LCS6-GG genotype; NA11831, LCS6-GT genotype; and NA11892, LCS6-TT genotype) and one negative control (no genomic DNA). Both genotyping control samples and negative control were duplicated across all plates. In addition, approximately 10% of patient DNA samples ($n = 280$) were randomly selected for duplication across tested DNA plates to ensure consistent calling. Patients with either the GG or GT genotypes were classified as carriers of the LCS6 variant, whereas patients with the TT genotype were classified as LCS6 WT.

Statistical analysis

All statistical analyses of the LCS6 variant used genotype data obtained from whole blood. The primary objective was to assess the prognostic value of LCS6 status in terms of DFS and time to recurrence (TTR). DFS was defined as the time from the date of randomization to the first documented disease recurrence or death from any causes. TTR was defined as time from the date of randomization to the first documented disease recurrence. For patients who died without recurrence, TTR was censored at the last disease evaluation date. Both DFS and TTR were censored at 4 years or last follow-up, whichever was earlier. χ^2 and unequal

variance two-sample *t* tests were used to compare categorical and continuous baseline factors, respectively, between patients carrying the LCS6 variant (GG or GT) and patients with LCS6 WT (TT; refs. 27, 28). Logistic regression was used to assess the association between LCS6 status and clinical outcomes (28). The method of Kaplan–Meier was used to estimate the distributions of DFS and TTR (29). Cox model was used to assess the univariate and multivariate associations between LCS6 and clinical outcomes (30). Unless otherwise specified, all multivariate models were adjusted for age, sex, race, performance score, stratification factors (T/N stage and grade), primary tumor site, treatment, and *KRAS*, *BRAF*, and MMR status. The interaction between LCS6 and *KRAS*, *BRAF*, and MMR status were assessed by Cox model with corresponding interaction terms. All analyses were performed in SAS v9 and conducted by the Alliance Statistics and Data Center.

Results

LCS6 genotype in blood DNA and tumor DNA

KRAS LCS6 genotyping was performed on 2,834 blood samples with the finding that 432 of 2,834 (15.2%) were heterozygous (GT, 14.6%, $n = 413$) or homozygous (GG, 0.7%, $n = 19$) for the LCS6 G-allele (LCS6 variant), and 2,402 of 2,834 (84.8%) were homozygous (TT) for the LCS6 T-allele (LCS6 WT). *KRAS* LCS6 genotyping was also performed in 977 tumor samples (paired with the corresponding blood samples) of which 143 of 977 (14.6%) were heterozygous (GT, 14.0%, $n = 137$) or homozygous (GG, 0.6%, $n = 6$) for the LCS6 G-allele, and 834 of 977 (85.4%) were homozygous (TT) for the LCS6 T-allele. Results for blood and tumor samples were highly concordant (99.8%) with discrepant results identified in samples from 2 patients (sample 1: TT/blood and GT/tumor; sample 2: GT/blood and GG/tumor). Repeating the LCS6 genotyping assay for both whole blood and tumor-derived DNA from the two discrepant samples showed identical results.

LCS6 variant, patient demographic, and clinicopathologic variables

The median age for both LCS6 variant and WT carriers was 58 years. Among the study population, 53.2% were male and 87.5% were Caucasian. The frequency of the LCS6 variant was 17.2% in Caucasian, 3.1% in Black or African-American, and 0.8% in Asian patients. G-allele carriers were significantly more frequent in Caucasians than in other races (χ^2 test, $P < 0.0001$). No statistically significant differences were found between LCS6 variant carriers and LCS6 WT carriers for age, sex, or study treatment arm (all $P > 0.1$, Table 1). In addition, no associations were found

Table 1. Patient demographics by *KRAS*-LCS6 status

	Carrier ($N = 432$)	WT ($N = 2,402$)	Total ($N = 2,834$)	*P*
Age, y				0.39[a]
N	432 (15.2)	2,402 (84.8)	2,834 (100.0)	
Median	58.00	58.00	58.00	
Range	(22.00–85.00)	(19.00–86.00)	(19.00–86.00)	
Age, *n* (%)				0.18[b]
<50	89 (13.6)	566 (86.4)	655 (23.1)	
≥ 50	343 (15.7)	1,836 (84.3)	2,179 (76.9)	
Sex, *n* (%)				0.91[b]
Female	201 (15.2)	1,125 (84.8)	1,326 (46.8)	
Male	231 (15.3)	1,277 (84.7)	1,508 (53.2)	
Race, *n* (%)				<0.0001[b]
Caucasian	419 (17.2)	2,021 (82.8)	2,440 (87.5)	
Black or African-American	6 (3.1)	190 (96.9)	196 (7.0)	
Asian	1 (0.8)	131 (99.2)	132 (4.7)	
Other	0 (0.0)	22 (100.0)	22 (0.8)	
Missing	6	38	44	
Treatment arms, *n* (%)				0.78[b]
FOLFOX	177 (15.1)	997 (84.9)	1,174 (41.4)	
FOLFIRI	15 (17.2)	72 (82.8)	87 (3.1)	
FOLFOX × 6 → FOLFIRI × 6	12 (12.4)	85 (87.6)	97 (3.4)	
FOLFOX + C225	168 (14.9)	956 (85.1)	1,124 (39.7)	
FOLFIRI+ C225	3 (10.0)	27 (90.0)	30 (1.1)	
FOLFOX × 6 → FOLFIRI × 6 + C225	6 (19.4)	25 (80.6)	31 (1.1)	
Treatment per local physician discretion	51 (17.5)	240 (82.5)	291 (10.3)	

[a]Unequal variance two-sample *t* test.
[b]χ^2 test.

Table 2. Patient clinicopathologic characteristics and genetic biomarkers by LCS6 status

	Carrier ($N = 432$)	WT ($N = 2,402$)	Total ($N = 2,834$)	*P*
T stage, *n* (%)				0.22[a]
T1 or T2	75 (17.2)	361 (82.8)	436 (15.4)	
T3 or T4	357 (14.9)	2,040 (85.1)	2,397 (84.6)	
Missing	0	1	1	
Number of positive LNs, *n* (%)				0.24[a]
1–3	246 (14.6)	1,440 (85.4)	1,686 (59.5)	
≥ 4	186 (16.2)	962 (83.8)	1,148 (40.5)	
Grade, *n* (%)				0.94[a]
High	105 (15.2)	588 (84.8)	693 (24.5)	
Low	327 (15.3)	1,814 (84.7)	2,141 (75.5)	
PS, *n* (%)				0.63[a]
PS 0	335 (15.4)	1,834 (84.6)	2,169 (76.6)	
PS 1 or 2	97 (14.7)	564 (85.3)	661 (23.4)	
Missing	0	4	4	
Site of disease, *n* (%)				0.21[a]
Right	219 (15.5)	1,197 (84.5)	1,416 (50.2)	
Left	208 (15.2)	1,156 (84.8)	1,364 (48.4)	
Both	2 (5.1)	37 (94.9)	39 (1.4)	
Missing	3	12	15	
Site of disease, *n* (%)				0.81[a]
Missing	26	149	175	
Cecum	93 (16.2%)	482 (83.8%)	575 (21.6%)	
Ascending colon	57 (13.7%)	359 (86.3%)	416 (15.6%)	
Hepatic flexure	15 (12.9%)	101 (87.1%)	116 (4.4%)	
Transverse colon	36 (16.9%)	177 (83.1%)	213 (8.0%)	
Splenic flexure	16 (15.1%)	90 (84.9%)	106 (4.0%)	
Descending colon	26 (18.1%)	118 (81.9%)	144 (5.4%)	
Sigmoid colon	163 (15.0%)	926 (85.0%)	1,089 (41.0%)	
Bowel obstruction, *n* (%)				0.77[a]
Yes	72 (15.7)	387 (84.3)	459 (16.2)	
No	360 (15.2)	2,015 (84.8)	2,375 (83.8)	
Bowel perforation, *n* (%)				0.67[a]
Yes	20 (14.0)	123 (86.0)	143 (5.0)	
No	412 (15.3)	2,279 (84.7)	2,691 (95.0)	
BMI, *n* (%)				0.14[a]
Underweight (BMI < 20)	10 (8.5)	107 (91.5)	117 (4.1)	
Normal weight (20$\leq$ BMI<25)	114 (15.6)	617 (84.4)	731 (25.9)	
Overweight (25$\leq$ BMI<30)	147 (14.6)	863 (85.4)	1,010 (35.8)	
Obese (BMI $\geq$ 30)	158 (16.4)	806 (83.6)	964 (34.2)	
Missing	3	9	12	
KRAS, *n* (%)				0.88[a]
Missing	13	84	97	
Mutant	150 (15.2)	839 (84.8)	989 (36.1)	
WT	269 (15.4)	1,479 (84.6)	1,748 (63.9)	
BRAF, *n* (%)				0.33[a]
Missing	20	139	159	
Mutant	58 (17.2)	279 (82.8)	337 (12.6)	
WT	354 (15.1)	1,984 (84.9)	2,338 (87.4)	
MMR, *n* (%)				0.68[a]
Missing	12	81	93	
pMMR	375 (15.4)	2,056 (84.6)	2,431 (88.7)	
dMMR	45 (14.5)	265 (85.5)	310 (11.3)	

Abbreviations: LNs, lymph nodes; PS, performance score; BMI, body mass index; MMR, mismatch repair.
[a]χ^2 test.

between the LCS6 genotype (variant vs. WT) and T stage, number of positive lymph node, tumor differentiation, performance status, primary tumor site, bowel obstruction or perforation, or BMI (all $P > 0.1$, Table 2).

Association of the LCS6 variant with *KRAS*, *BRAF*, and MMR status

The overall frequencies of *KRAS* mutant, *BRAF* mutant, and dMMR tumors were 36.1%, 12.6%, and 11.3%, respectively. No statistically significant differences were found between LCS6 variant and WT carriers *for KRAS*, *BRAF*, or MMR status (all $P > 0.1$, Table 2).

Prognostic impact of the LCS6 genotype

The 3-year DFS rate was 74.1% (number of events = 104; 95% confidence interval, CI, 69.5%–78.7%) and 72.5% (number of events = 606; 95% CI, 70.5%–74.5%) in LCS6 variant and WT carriers, respectively (log-rank test, $P = 0.49$, Fig. 1A). The 3-year recurrence-free survival rate was 75.7% (number of events = 93; 95% CI, 71.2%–80.3%) and 74.5% (number of events=549; 95% CI, 72.6%–76.5%) in LCS6 variant and WT carriers, respectively (log-rank test, $P = 0.43$, Fig. 1B). Within LCS6 variant and WT carriers, no statistically significant differences were found in DFS (HR, 0.93; 95% CI, 0.76–1.14, Fig. 1A) or TTR (HR, 0.92; 95% CI, 0.74–1.14, Fig. 1B). Similar results were obtained after adjusting for age, sex, race, performance score, T/N stage, grade, primary tumor site, KRAS mutation, BRAF mutation, MMR status, and treatment (DFS: HR, 0.885; 95% CI, 0.711–1.102; *P*, 0.2759 and TTR: HR, 0.870; 95% CI, 0.689–1.097; *P*, 0.2385). Cox model analysis for the individual LCS6 genotypes (GG vs. GT vs. TT) also showed no significant associations with either DFS ($P = 0.5738$) or TTR ($P = 0.6713$). No significant interaction effect was shown between the LCS6 variant and treatment arm on DFS ($P = 0.2401$) or TTR ($P = 0.2495$). Further analysis within specific treatment arms also showed no statistically significant associations between the LCS6 variant and DFS. In an analysis of the LCS6 genotype in relation to the status of *KRAS* (Fig. 2A), *BRAF* (Fig. 2B), or MMR (Fig. 2C), no statistically significant differences in DFS were found (Table 3). In addition, the LCS6 variant showed no significant interaction effect with *KRAS* mutation status ($P = 0.42$), *BRAF* mutation status ($P = 0.16$), MMR status ($P = 0.84$), or tumor site ($P = 0.6616$).

Discussion

Previous studies have established let-7 as a tumor suppressor miRNA, which negatively regulates the RAS pathway (14, 16, 17). In 2008, Chin and colleagues reported on a polymorphism in a let-7 miRNA complementary site 6 in the KRAS 3′ (LCS6) that showed a significant association with increased risk for non–small cell lung carcinoma (NSCLC) among moderate smokers (18). Since then, the LCS6 polymorphism has been studied extensively in other cancer types, such as oral cavity, ovarian, colorectal, and breast (21, 26, 31, 32). However, the clinical significance of the LCS6 polymorphism in different cancer types and among different stages within CRC has been inconsistent. To evaluate the significance of LCS6 variant in colon cancers, we focused on patients with stage III cancer from a large, prospectively randomized clinical trial of adjuvant chemotherapy. Our association study indicates that the germline LCS6 genotype was not associated with *KRAS* mutation status or with clinical outcome in patients with stage III colon cancers.

Our study confirms that the LCS6 variant is a germline polymorphism with genotypes that were highly concordant

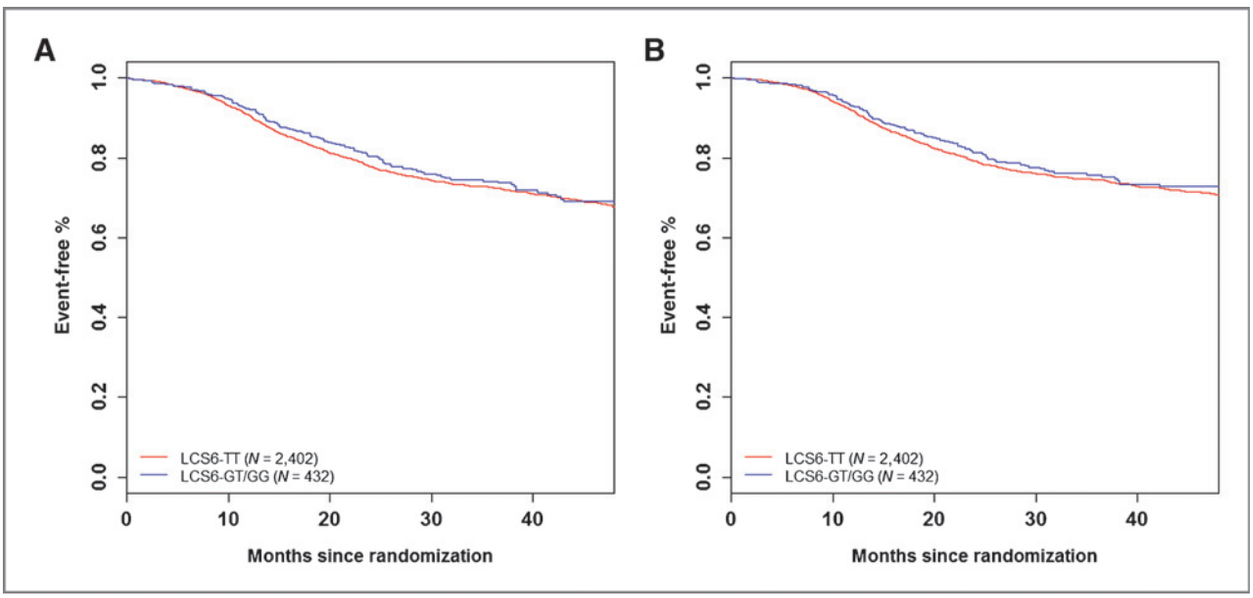

Figure 1. Univariate association of the *KRAS*-LCS6 variant with DFS (A) and TTR (B) in patients with stage III colon cancer.

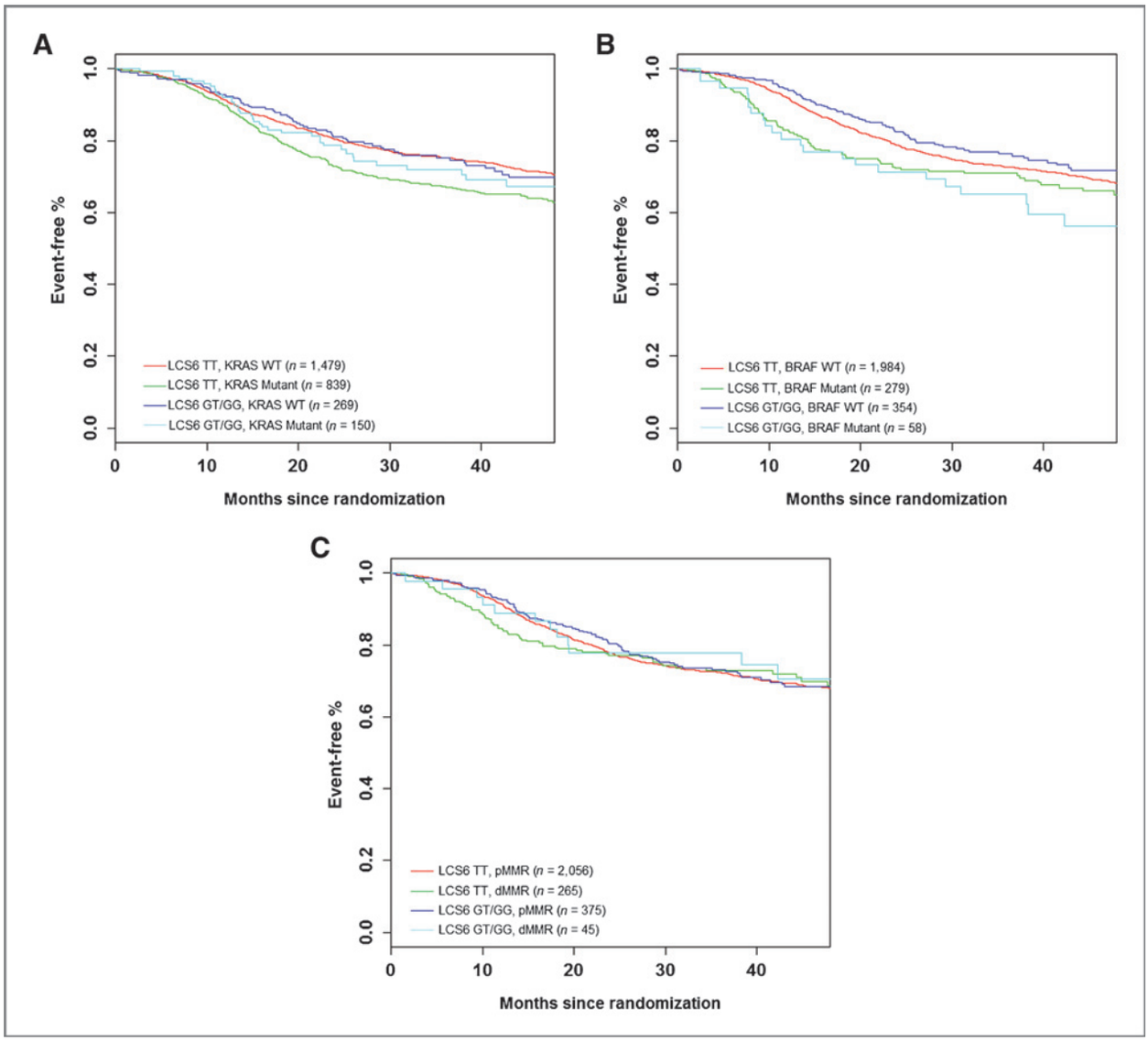

Figure 2. Impact of *KRAS* mutations (A), *BRAF*^{V600E} (B), and DNA MMR (C) status on DFS according to *KRAS*-LCS6 variant status.

(99.8%) in paired blood and tumor DNA. Similar to our findings, Sebio and colleagues found a concordance rate of 98%, with two blood DNA samples displaying the LCS6 genotype TG, whereas the two paired tumor DNA samples showed the LCS6 genotype TT (23). Though a rare occurrence, blood versus tumor DNA discrepancies could result from various events such as loss of heterozygosity in tumor samples, cross-contamination in tissue sampling, DNA fragmentation during the formalin-fixation and paraffin-embedding processing, or artifactual nucleotide substitutions from problematic PCR amplification (33, 34).

Our study identified a significantly higher frequency of the LCS6 G-allele carriers in Caucasians compared with other races, which is consistent with the published minor allele frequencies (MAF) available from the 1,000 Genomes Project (Caucasians MAF = 0.086; African MAF = 0.004; ref. 35). Importantly, racial differences in CRC incidence and mortality exist among Caucasian and African-American populations (36) with African Americans being more likely to be diagnosed at a younger age, with late-stage disease, proximal tumors, and worse prognosis compared with Caucasians (37). To date, however, the biologic and genetic basis for the existence of a more aggressive CRC phenotype in African Americans awaits further study.

Our analysis showed no associations between the LCS6 variant and either tumor localization, specific tumor subsites, or *KRAS* somatic mutation status. Tumor location has been shown to display distinct differences in molecular characteristics. Previous studies indicated *KRAS*-mutated carcinomas were more frequently located in the proximal compared with distal CRC (38). In addition, cecal cancers

Table 3. Association between the LCS6-variant and DFS stratified by *KRAS*, *BRAF*, and MMR status

	HR (95% CI)	*P*
KRAS mutation status		
LCS6 TT, KRAS WT ($n = 1,479$)	0.73 (0.62–0.86)	0.002
LCS6 TT, KRAS mutant ($n = 839$)	Ref. (Ref.)	Ref.
LCS6 GT/GG, KRAS WT ($n = 269$)	0.73 (0.55–0.96)	0.025
LCS6 GT/GG, KRAS mutant ($n = 150$)	0.83 (0.59–1.18)	0.304
BRAF mutation status		
LCS6 TT, BRAF WT ($n = 1,984$)	1.18 (0.93–1.51)	0.17
LCS6 TT, BRAF mutant ($n = 279$)	1.47 (1.08–2.00)	0.015
LCS6 GT/GG, BRAF WT ($n = 354$)	Ref. (Ref.)	Ref.
LCS6 GT/GG, BRAF mutant ($n = 58$)	1.81 (1.13–2.91)	0.015
MMR status		
LCS6 TT, pMMR ($n = 2,056$)	1.09 (0.61–1.92)	0.78
LCS6 TT, dMMR ($n = 265$)	1.13 (0.61–2.08)	0.70
LCS6 GT/GG, pMMR ($n = 375$)	1.03 (0.56–1.88)	0.93
LCS6 GT/GG, dMMR ($n = 45$)	Ref. (Ref.)	Ref.

have also exhibited the highest frequency of *KRAS* mutations (39). In agreement with our findings, previous reports have also shown no correlation between the LCS6 variant and *KRAS* mutation status in both colon cancer (19) and NSCLC (40). These results suggest that LCS6 and *KRAS* somatic mutation status are independent events. A possible explanation is that *KRAS* upregulation accompanying the LCS6 variant does not result in any selective pressure for or against *KRAS* mutation (40). However, Graziano and colleagues reported a conflicting result showing a significantly greater frequency of LCS6 G-allele carriers in the *KRAS* mutation group compared with the *KRAS* WT group in patients with metastatic CRC (21). It is hypothesized that some clonal selection in tumors may occur, favoring less differentiated and more aggressive clones that harbor both activating *KRAS* mutations and LCS6. Though the role of LCS6 variant in *KRAS* mutation remains to be delineated, reported association discrepancies may be explained by the heterogeneity in tumor pathologic type and stage, study design, or sample size.

In the current study, we failed to detect any significant association between the LCS6 polymorphism and survival in patients with stage III colon cancer, even after combining LCS6 genotype with mutation status of either *KRAS* or *BRAF*, or with MMR status. Conflicting data exist about this polymorphism in other stages of CRC. In this regard, a significantly better survival was reported in LCS6 G-allele carriers that was enhanced when combined with *KRAS*-mutant status in early-stage (stage I and II, $n = 409$), but not in later-stage (stage III, $n = 182$ and stage IV, $n = 69$) CRCs (19). However, Ryan and colleagues recently showed associations between the LCS6 G-allele and reduced risk of mortality in patients with late-stage (stage III and IV, $n = 124$), but not in early-stage (stage I and II, $n = 113$) CRC (22). Controversy also exists about the role of LCS6 polymorphism in prognosis of other solid tumors. A reduced survival was reported in patients with oral cancer (26), yet no association between the LCS6 polymorphism and survival was found in NSCLC (40) or ovarian cancer (32). The conflicting evidence about the prognostic value of the LCS6 variant may be attributed to multiple factors: differences in study design, inadequate statistical power, selection bias, and heterogeneity within cancer stages and cancer types.

Our analysis also identified no interaction effect for the LCS6 variant and treatment arm (FOLFOX alone versus FOLFOX and cetuximab) and showed no associations between LCS6 variant status and DFS within the separate treatment groups. Conflicting evidence also exists for the LCS6 variant as a predictive biomarker in *KRAS* WT CRC patients treated with cetuximab. In patients treated with salvage cetuximab-irinotecan therapy, significant associations were found between carriers of the LCS6 G-allele and adverse PFS and overall survival (OS; ref. 21). However, conflicting results were reported in patients with metastatic CRC treated with cetuximab monotherapy with LCS6 WT (TT) patients showing a significantly decreased tumor response, but no association between LCS6 genotype and PFS or OS regardless of *KRAS* status (20). Most recently, Sebio and colleagues identified a significant decrease in tumor response rate in LCS6 G-allele carriers with refractory mCRC; however, there was no significant association between the LCS6 variant and PFS or OS (23). This association was identified only in patients treated with anti–EGFR-based therapy either alone or in combination, not in patients treated with FOLFIRI alone. Although the aforementioned studies were conducted in patients with treatment refractory disease, the Nordic trial was conducted in previously untreated patients with metastatic CRC. In this study, there was no statistically significant effect of the LCS6 variant allele on response rate, PFS, or OS in patients treated with FLOX ± cetuximab (41).

Strengths of our study include the large number of paired blood and tumor specimens that were prospectively collected, analyzed at a single institution, and from a clinical

trial with meticulous data collection, including recurrence and survival. We examined a uniform population of stage III colon cancers as compared with studies that include a mixture of stages with small sample sizes. To our knowledge, our study is the largest conducted to date that examines the LCS6 polymorphism in patients with CRC with sufficient statistical power to detect the association between LCS6 variant, *KRAS* mutation status, and disease outcome. However, our study has some limitations. Our trial cohort represents a highly selected group of patients with stage III colon cancer through strict inclusion criteria. Thus, bias is unavoidable, and generalizability of our findings needs to be proved in colon cancer with other stages (stage I, II, and IV) and other cancer types. In addition, *KRAS* mutation profiling in the N0147 study population remains incomplete. Previous reports have indicated that *KRAS* mutations in codon 61 and 146 may potentially predict resistance to cetuximab in *KRAS* codon 12 and 13 wild-WT metastatic colorectal cancer (42). Furthermore, our adjuvant clinical trial population of patients with stage III colon cancer is also unable to assess the potential association of the LCS6 variant with tumor response, although recurrence and survival were studied.

In conclusion, we report the largest association study investigating the LCS6 polymorphism and colon cancer outcome. We found that the LCS6 polymorphism is not associated with *KRAS* mutation status or with disease outcome in patients with stage III colon cancer. However, the clinical utility of the LCS6 polymorphism in other stages of colon cancer is poorly understood and awaits further study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

Conception and design: D. Sha, A. Lee, Q. Shi, S.R. Alberts, D.J. Sargent, F.A. Sinicrope
Development of methodology: D. Sha, Q. Shi, D.J. Sargent
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Sha, A. Lee, S.R. Alberts, F.A. Sinicrope
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Sha, Q. Shi, D.J. Sargent, F.A. Sinicrope
Writing, review, and/or revision of the manuscript: D. Sha, A. Lee, Q. Shi, S.R. Alberts, D.J. Sargent, F.A. Sinicrope, R.B. Diasio
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Sha, A. Lee, D.J. Sargent
Study supervision: D. Sha, S.R. Alberts, D.J. Sargent, F.A. Sinicrope, R.B. Diasio

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