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Expanded Low Allele Frequency *RAS* and *BRAF* V600E Testing in Metastatic Colorectal Cancer as Predictive Biomarkers for Cetuximab in the Randomized CO.17 Trial

Jonathan M. Loree[1], Anthony Dowers[2], Dongsheng Tu[3], Derek J. Jonker[4], Daniel L. Edelstein[5], Hannah Quinn[5], Frank Holtrup[5], Timothy Price[6], John R. Zalcberg[7], Malcolm J. Moore[1], Christos S. Karapetis[8], Chris J. O'Callaghan[3], Paul Waring[2], Hagen F. Kennecke[9], Stanley R. Hamilton[10], and Scott Kopetz[11]

ABSTRACT

Purpose: Expanded *RAS/BRAF* mutations have not been assessed as predictive for single-agent cetuximab in metastatic colorectal cancer (mCRC), and low mutant allele frequency (MAF) mutations are of unclear significance. We aimed to establish cetuximab efficacy in optimally selected patients using highly sensitive beads, emulsion, amplification, and magnetics (BEAMing) analysis, capable of detecting alterations below standard clinical assays.

Patients and Methods: CO.17 trial compared cetuximab versus best supportive care (BSC) in *RAS/BRAF*-unselected mCRC. We performed *RAS/BRAF* analysis on microdissected tissue of 242 patients in CO.17 trial using BEAMing for *KRAS/NRAS* (codons 12/13/59/61/117/146) and *BRAF* V600E. Patients without BEAMing but with previous Sanger sequencing–detected mutations were included.

Results: *KRAS*, *NRAS*, and *BRAF* mutations were present in 53%, 4%, and 3% of tumors, respectively. Cetuximab improved overall survival [OS; HR, 0.51; 95% confidence interval (CI), 0.32–0.81; $P = 0.004$] and progression-free survival (PFS; HR, 0.25; 95% CI, 0.15–0.41; $P < 0.0001$) compared with BSC in *RAS/BRAF* wild-type patients. Cetuximab did not improve OS/PFS for *KRAS*-, *NRAS*-, or *BRAF*-mutated tumors, and tests of interaction confirmed expanded *KRAS* ($P = 0.0002$) and *NRAS* ($P = 0.006$) as predictive, while *BRAF* mutations were not ($P = 0.089$). BEAMing identified 14% more tumors as *RAS* mutant than Sanger sequencing, and cetuximab lacked activity in these patients. Mutations at MAF < 5% were noted in 6 of 242 patients (2%). One patient with a *KRAS* A59T mutation (MAF = 2%) responded to cetuximab. More *NRAS* than *KRAS* mutations were low MAF (OR, 20.50; 95% CI, 3.88—96.85; $P = 0.0038$).

Conclusions: We establish single-agent cetuximab efficacy in optimally selected patients and show that subclonal *RAS/BRAF* alterations are uncommon and remain of indeterminate significance.

Introduction

The anti-EGFR antibodies, cetuximab and panitumumab, are important treatment options for patients with metastatic colorectal cancer (mCRC). *KRAS/NRAS* (*RAS*) mutation status and primary tumor location guide treatment selection, with left-sided *RAS* wild-type tumors showing greatest benefit from anti-EGFR antibodies (1–7). Patients with *BRAF* V600E mutations may also have reduced benefit from anti-EGFR therapy (8). However, it is unclear whether *BRAF* mutations obviate all benefits, and a test of interaction for the predictive utility of *BRAF* V600E mutations has not been established.

Although *RAS* and *BRAF* V600E sequencing helps identify the optimal population to treat with anti-EGFR antibodies, eventually patients develop resistance through acquired *RAS* mutations, which appear to be expanded from rare clones preexisting in the tumor (9). Longitudinal assessment of circulating tumor DNA (ctDNA) has provided evidence that resistant clones decay with time, creating an opportunity for anti-EGFR rechallenge (10). In the CRICKET phase II trial, patients previously treated with anti-EGFR antibodies who subsequently progressed were rechallenged with cetuximab + irinotecan after intervening therapy. Among patients who were *RAS* wild-type by ctDNA preceding rechallenge, progression-free survival (PFS) was 4 months (11). Numerous other rechallenge protocols are underway, many with drug combinations, and it is essential to understand the magnitude of benefit of single-agent anti-EGFR therapy to establish a bench mark for rechallenge (9). Expanded *RAS* and *BRAF* V600E have not been previously assessed in a randomized single-agent cetuximab trial to establish predictive capacity and their utility is extrapolated from multi-agent or panitumumab trials.

It is also unclear whether mutations below the 5% mutant allele frequency (MAF) limit of detection of standard assays are of importance (12). Although historic PCR and Sanger sequencing methods identified mutations occurring at MAFs above 10%–20%, newer techniques have sensitivities down to 0.1% and may lead to improved outcomes (13). In the CAPRI-GOIM trial evaluating FOLFIRI +

[1]BC Cancer, University of British Columbia, Vancouver, British Columbia, Canada. [2]The University of Melbourne, Melbourne, Victoria, Australia. [3]Canadian Cancer Trials Group, Kingston, Ontario, Canada. [4]The Ottawa Hospital, University of Ottawa, Ottawa, Ontario, Canada. [5]Sysmex-Inostics, Baltimore, Maryland. [6]Queen Elizabeth Hospital, Adelaide, South Australia, Australia. [7]Monash University, Melbourne, Victoria, Australia. [8]Flinders University, Adelaide, South Australia, Australia. [9]Virginia Mason Cancer Institute, Seattle, Washington. [10]City of Hope, Los Angeles, California. [11]University of Texas, MD Anderson Cancer Center, Houston, Texas.

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Corresponding Author: Scott Kopetz, University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 426, Houston, TX 77030. Phone: 713-792-2828; Fax: 713-563-6764; E-mail: skopetz@mdanderson.org

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Translational Relevance

The predictive utility of expanded *RAS* and *BRAF* for anti-EGFR therapy in colorectal cancer arises from panitumumab trials, and it is unclear whether low mutant allele frequency (MAF) mutations in these genes impact efficacy. We evaluated tissues from the CO.17 trial that randomized patients to cetuximab or best supportive care with a high-sensitivity assay (beads, emulsion, amplification, and magnetics analysis, BEAMing) and microdissection for expanded *RAS/BRAF* mutations (MAF > 1%). Cetuximab improved overall and progression-free survival in patients with *RAS/BRAF* V600E wild-type tumors relative to supportive care, and a test of interaction confirmed *RAS* ($P < 0.01$) but not *BRAF* ($P = 0.089$) mutations as predictive for cetuximab benefit. BEAMing showed increased sensitivity and identified 14% more *KRAS* mutations than historic Sanger sequencing. Mutations in *RAS* with allele frequency <5% were noted in 2% of patients, one of whom responded to cetuximab.

cetuximab in mCRC, next-generation sequencing revealed an additional 15.9% of patients with *KRAS* exon 2 mutations beyond Sanger sequencing. These patients had inferior outcomes compared with patients with *RAS* wild-type tumors and similar prognosis to high allele frequency *RAS* mutations (14). These findings have been replicated by many retrospective studies, however, it remains unclear whether low allele frequency mutations obviate all benefits (15–17). In the CRYSTAL trial evaluating cetuximab and FOLFIRI in the first line, the use of high sensitivity beads, emulsion, amplification, and magnetics (BEAMing) analysis demonstrated a relationship between the *RAS* MAF and anti-EGFR efficacy and it was unclear whether low MAF mutations prevent all benefits (18).

Given the current gaps in knowledge, we undertook a retrospective analysis of the CO.17 trial comparing cetuximab with best supportive care (BSC) to establish (i) the efficacy of single-agent cetuximab in optimally selected *RAS/BRAF* wild-type patients relative to BSC and (ii) the frequency and clinical relevance of low allele frequency *RAS* mutations detected by an ultra-sensitive assay.

Patients and Methods

Patient population

CO.17 was a phase III clinical trial that randomized patients 1:1 to receive either cetuximab or BSC after institutional review board (IRB) approval (NCT00079066; ref. 1). The study was IRB approved, with written consent obtained from all subjects, and conducted in accordance with the Declaration of Helsinki and International Ethical Guidelines for Biomedical Research Involving Human Subjects. Patients consented to enrollment and correlative studies and had either progressed on or were intolerant to fluoropyrimidine, oxaliplatin, and irinotecan. No prior anti-EGFR therapy was allowed. Enrollment was unselected for *RAS/BRAF*.

This correlative analysis assessed all patients with remaining evaluable tissue ($N = 242$). Median time from tissue collection to randomization was 2.2 years for patients who underwent analysis with BEAMing and did not differ between arms of the study ($P = 0.22$). Of 211 samples with known site of origin, 207 arose from primary tumors (98.1%), while four were from metastases (1.9%). There were 84 patients without remaining tissue that were historically identified to have a *KRAS* exon 2 ($N = 76$) or *BRAF* V600E ($N = 8$) mutation using Sanger sequencing from previously published analyses that were included (1, 19). Two additional patients were not analyzable for *KRAS* in the BEAMing assay, but historically had a *KRAS* exon 2, which was used to fill in the missing result. Patients with a prior mutation were included, however, patients with no remaining tissue and a prior result that did not identify a mutation were excluded, as the previous assessments lacked coverage of all *KRAS/NRAS* codons.

Treatment

Cetuximab treatment consisted of an intravenous loading dose of 400 mg/m^2 followed by 250 mg/m^2 given weekly until progression.

RAS and BRAF testing

Archival formalin-fixed, paraffin-embedded (FFPE) blocks were evaluated for sample quality prior to sectioning five slides for DNA extraction. Areas of highest tumor content were selected and microdissected. DNA was extracted using QIAMP DNA FFPE tissue kits with barcoding to maintain sample continuity.

Prior to sequencing, samples underwent a repair step using the New England Biolabs PreCR Repair Mix. DNA isolated from FFPE blocks was subjected to LINE-1 qPCR for quantification and quality control (20). Only PCR accessible, inhibition-free, and amplifiable target regions qualified for subsequent analysis. For any sample with amplicons exhibiting insufficient amplification, PCR products underwent additional analysis on an agarose gel to confirm successful and target-specific amplification before BEAMing analysis was performed. Two of 242 samples had one or more amplicons in *RAS* that were not analyzable due to unamplifiable DNA. These samples were still analyzed for *BRAF* mutations.

A previously described, highly sensitive BEAMing analysis was utilized to detect mutations in *KRAS/NRAS* (codons 12, 13, 59, 61, 117, and 146) and *BRAF* V600E with coverage outlined in Supplementary Table S1 and a 1% MAF limit of detection. Sequencing was carried out by Sysmex Inostics (15, 18, 21).

Statistical analysis

Survival was summarized with Kaplan–Meier curves and compared using stratified log-rank tests adjusted for performance status at randomization. HRs and 95% confidence intervals (95% CI) were calculated from stratified Cox regression models with treatment group as the single factor. Overall survival (OS) was defined as the time from randomization until death from any cause. PFS was defined as the time from randomization until progression or death from any cause. To determine whether expanded *RAS* and *BRAF* V600E mutations were predictive, we used a Cox model with treatment, mutation status, and their interaction term as covariates. Objective response rate (ORR) was defined according to modified RECIST (22). Between group comparisons used Kruskal–Wallis tests for continuous variables or a χ^2/Fisher exact test as appropriate.

Results

Patient population

Of 572 patients, 242 (42%) underwent analysis with BEAMing. BEAMing was successful in all samples for *BRAF*, but three had inconclusive *RAS* analysis. Baseline characteristics are summarized in **Table 1**. Prevalence in the BEAMing population was 97 (41%) *RAS/BRAF* V600E wild-type, 126 (53%) *KRAS*, nine (4%) *NRAS*, and seven (3%) *BRAF* V600E mutated, with specific mutations noted in Supplementary Table S2. There were 5 patients with two concurrent *KRAS* mutations, while 1 patient had three concurrent *KRAS*

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Table 1. Baseline patient characteristics.

Characteristic	*RAS* and *BRAF* V600E wild-type ($n = 97$)	*KRAS* mutated ($n = 204$)	*NRAS* mutated ($n = 9$)	*BRAF* V600E mutated ($n = 15$)	*P*
Median age (range)	64 (29–88)	63 (37–86)	69 (47–75)	64 (39–77)	0.79
Gender					
Female	28 (29)	72 (35)	2 (22)	5 (33)	0.63
Male	69 (71)	132 (65)	7 (78)	10 (67)	
ECOG					
0	26 (27)	46 (23)	1 (11)	1 (7)	0.29
1	58 (60)	117 (57)	5 (56)	9 (60)	
2	13 (13)	41 (20)	3 (33)	5 (33)	
Side of tumor					
Right	18 (19)	71 (37)	3 (38)	10 (67)	0.0005
Left	78 (81)	123 (63)	5 (63)	5 (33)	
Prior treatment					
5-FU	97 (100)	204 (100)	9 (100)	15 (100)	1
Irinotecan	92 (95)	199 (98)	8 (89)	14 (93)	0.36
Oxaliplatin	96 (99)	201 (99)	9 (100)	15 (100)	0.93
Site of disease					
Liver	87 (90)	159 (78)	8 (89)	10 (67)	0.038
Lung	56 (58)	133 (65)	5 (56)	10 (67)	0.60
Nodes	51 (53)	80 (39)	3 (33)	7 (47)	0.16
Treatment					
Cetuximab	54 (56)	101 (50)	3 (33)	7 (47)	0.45
BSC	43 (44)	103 (51)	6 (67)	8 (53)	

Note: Percentages represent the % of known.
Abbreviations: 5-FU, 5-fluorouracil; ECOG, Eastern Cooperative Oncology Group; *N* = number.

mutations. Patients with multiple alterations frequently had second mutations of low allele frequency. These cases were excluded from analysis of low allele frequency variants as they had both high and low allele frequency alterations (Supplementary Fig. S1).

OS

OS was significantly improved with cetuximab compared with BSC (median, 10.1 vs. 4.8 months; HR, 0.51; 95% CI, 0.32–0.81; $P = 0.004$) in patients with *RAS/BRAF* V600E wild-type tumors (**Fig. 1A**). No improvement in OS was noted following cetuximab in patients with *KRAS*- (HR, 0.86; 95% CI, 0.63–1.16; $P = 0.32$), *NRAS*- (HR, 3.93; 95% CI, 0.65–23.89; $P = 0.11$), combined *RAS*- (HR, 0.91; 95% CI, 0.68–1.23; $P = 0.55$), or *BRAF* V600E–mutated tumors (HR, 0.71; 95% CI, 0.22–2.27; $P = 0.56$), compared with BSC (**Fig. 1B**). A test of interaction was positive for combined *RAS* ($P = 0.037$) and *NRAS* ($P = 0.026$), but not for *KRAS* alone ($P = 0.067$) or *BRAF* V600E mutations ($P = 0.24$), as predictive biomarkers for OS following cetuximab. Among *RAS/BRAF* V600E wild-type patients, left-sided tumors (median, 10.4 vs. 4.8 months; HR, 0.55; 95% CI, 0.33–0.91; $P = 0.019$) had improved OS with cetuximab relative to BSC, but this was not significant for right-sided tumors (median, 5.7 vs. 3.7 months; HR, 0.35; 95% CI, 0.11–1.07; $P = 0.055$). We repeated our analysis, but included patients with mutations MAF <5% as wild-type ($N = 6$) and noted no differences in results (Supplementary Table S3).

PFS

Among patients with *RAS/BRAF* V600E wild-type tumors, PFS improved following cetuximab relative to BSC (median, 5.4 vs. 1.8 months; HR, 0.25; 95% CI, 0.15–0.41; $P < 0.0001$; **Fig. 2A**). There did not appear to be any prolongation of PFS with the use of cetuximab for patients with *KRAS* (HR, 1.03; 95% CI, 0.78–1.35; $P = 0.86$), *NRAS* (HR, 1.26; 95% CI, 0.28–5.74; $P = 0.76$), combined *RAS* (HR, 1.04; 95% CI, 0.79–1.37; $P = 0.76$), or *BRAF* V600E mutations (HR, 0.75; 95% CI, 0.26–2.19; $P = 0.60$; **Fig. 2B**). *KRAS* ($P = 0.0002$), *NRAS* ($P = 0.006$), and combined *RAS* mutations ($P = 0.0001$) were predictive of lack of benefit from cetuximab for PFS using a test of interaction, while *BRAF* V600E mutations neared significance for predictive utility ($P = 0.089$). Left-sided *RAS/BRAF* V600E wild-type tumors had prolonged PFS following cetuximab (median, 5.5 vs. 2.0; HR, 0.20; 95% CI, 0.10–0.37; $P < 0.0001$), while right-sided tumors did not meet significance (median, 3.6 vs. 1.8 months; HR, 0.48; 95% CI, 0.17–1.36; $P = 0.16$). Similar to OS, when we categorized the 6 patients with mutations occurring at MAF < 5% as wild-type and repeated the analysis, we noted no change in the PFS endpoint (Supplementary Table S3).

Response rate

ORR (19% vs. 0%; $P = 0.002$) was significantly improved with cetuximab compared with BSC in *RAS/BRAF* V600E wild-type colorectal cancer (**Fig. 4**). Among patients with *KRAS* (ORR, 2%), *NRAS* (ORR, 0%), combined *RAS* (ORR, 2%), and *BRAF* V600 mutations (ORR, 0%) there was no difference in ORR relative to BSC, where ORR was 0% in all molecular groups. In left-sided *RAS/BRAF* V600E wild-type tumors, ORR was higher than right-sided tumors (23% vs. 0%; $P = 0.18$), but not significantly different. Categorizing mutations with MAF < 5% as wild-type did not change the ORR for patients with *RAS/BRAF* V600E wild-type tumors, but did decrease *KRAS* and combined *RAS* ORR to 1%.

Low MAF mutations

Low allele frequency mutations (MAF < 5%) occurred in 6 of 242 patients (2%). In these six tumors, three *KRAS* (G12V, A59T, and A59T) and three *NRAS* mutations (G13R, A146T, and A59T) were

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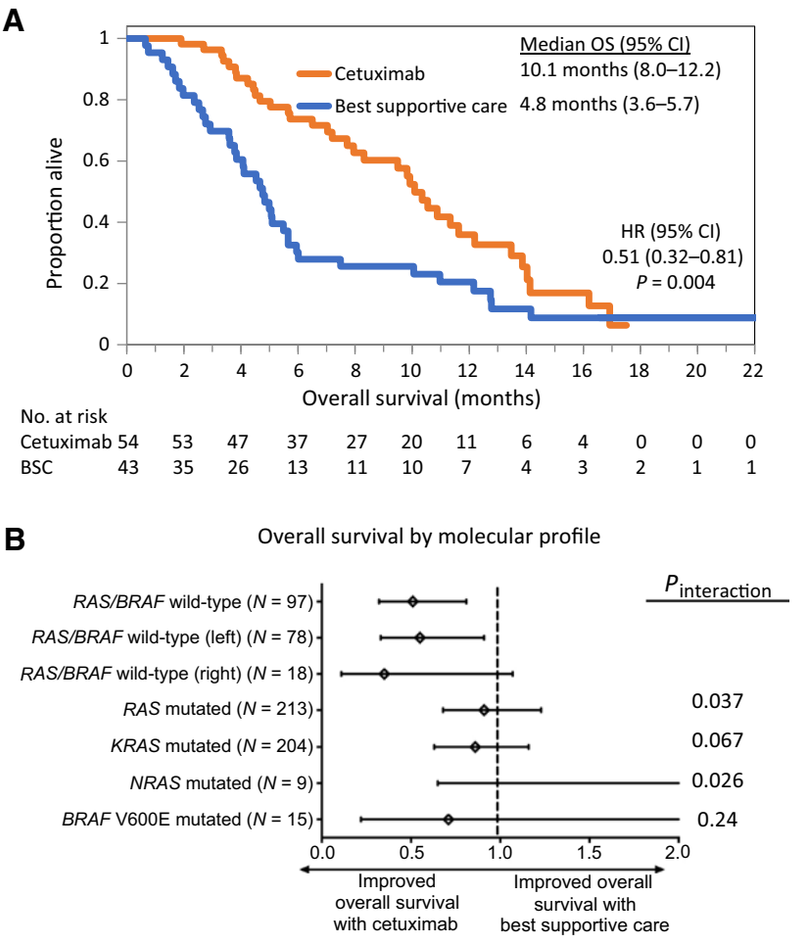

Figure 1.
Impact of cetuximab on OS in patients with *RAS/BRAF* V600E wild-type mCRC compared with BSC in the CO.17 trial (**A**) and stratified by molecular subgroup (**B**).

identified. Mutations in *NRAS* were more likely to occur at low allele frequency than *KRAS* (OR, 20.5; 95% CI, 3.9–96.9; $P = 0.0038$). A59T *RAS* mutations were present in 3 of 6 patients (2 with *KRAS* and 1 *NRAS*) with low MAF alterations compared with 0 of 136 patients with mutations occurring at MAF > 5% (OR, ∞; 95% CI, 26.28–∞; $P < 0.0001$).

Most mutations occurred at high allele frequencies consistent with a clonal mutation (**Fig. 3**). *KRAS* variants trended toward higher MAF than *NRAS* ($P = 0.058$), but did not differ from *BRAF* V600E ($P = 0.69$). *NRAS* and *BRAF* V600 allele frequencies did not differ ($P = 0.32$). There were 34 (14%) patients who had results for *KRAS* exon 2 available from Sanger sequencing who were previously wild-type, but now had a mutation in *KRAS* exon 2 detected with BEAMing. The median MAF for these 34 patients was 20% (range, 2%–60%) and treatment with cetuximab did not improve OS (median, 6.8 vs. 5.4 months; HR, 0.60; 95% CI, 0.27–1.35; $P = 0.21$), PFS (median, 1.9 vs. 1.8 months; HR, 0.72; 95% CI, 0.36–1.46; $P = 0.36$), or response rate (7% vs. 0% with BSC; $P = 0.41$) among these patients, suggesting they were clinically relevant. Seven patients (2.9%) previously had Sanger sequencing–detected *KRAS* mutations, but were reclassified as wild-type. All discordant cases had high quality assay results with BEAMing and were reviewed.

Of the 3 patients with *RAS* mutations at MAF < 5% who received cetuximab, 2 progressed after 2.7 and 3.7 months with only 1 patient having a partial response that lasted 11.2 months, while those with low MAF *RAS* mutations in the BSC arm progressed after 1.9 and 3.6 months, with 1 patient withdrawing and none having a response. The one response to cetuximab occurred in a male patient with a *KRAS* A59T mutation (MAF = 2%) occurring in a left-sided tumor. The patient had received prior fluoropyrimidine, oxaliplatin, and irinotecan and had liver-limited metastatic disease. OS for patients with low MAF *RAS* mutations was 11.6, 18.2, and 12.4 months following cetuximab and 2.7, 10.7, and 12.0 months following BSC.

Discussion

This updated analysis of CO.17 trial refines our understanding of the magnitude of benefit from single-agent cetuximab in optimally selected patients. Compared with the previous assessment of only *KRAS* exon 2 mutations [median PFS (mPFS) of 3.7 months with cetuximab], mPFS increased to 5.4 months with improved molecular profiling (1). Using highly sensitive BEAMing, we identified an additional 14% of patients who were wild-type by Sanger sequencing and lacked benefit from cetuximab, highlighting the utility of more sensitive assays. This work also enhances our knowledge of predictive biomarkers in mCRC. Previously, a test of interaction for anti-EGFR interacting with expanded *RAS* mutations was only available for panitumumab, not cetuximab (3). In addition, despite the nonsignificant ($P = 0.089$) test of interaction for *BRAF* V600E mutations being a predictive biomarker, this work highlights reduced benefit from anti-

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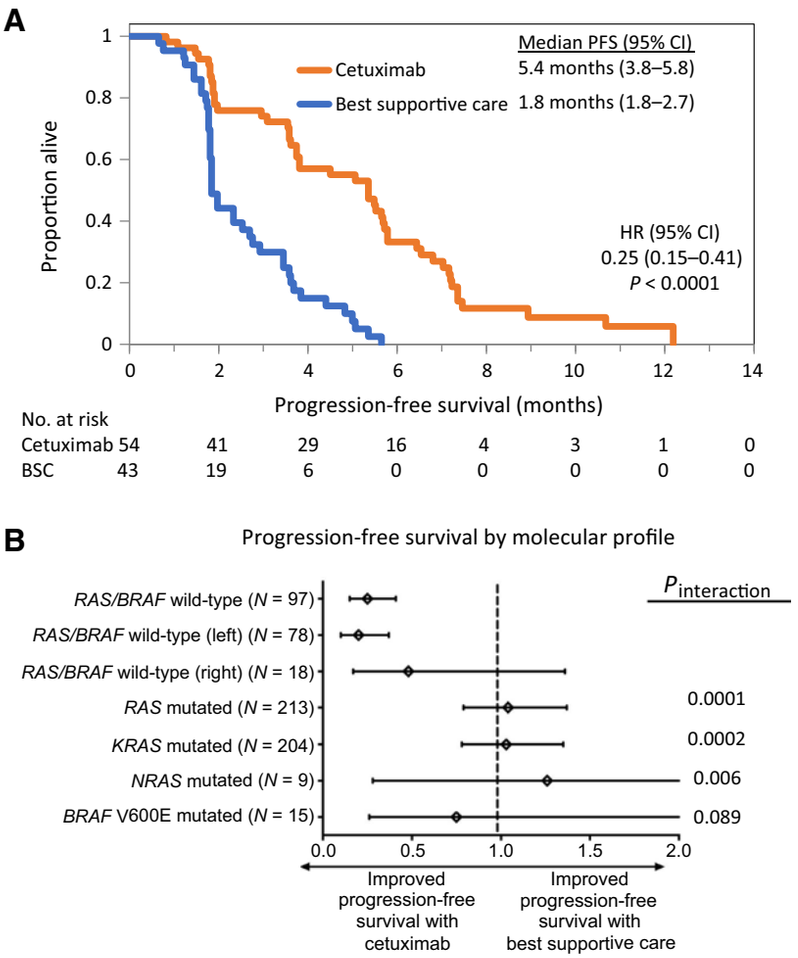

Figure 2.
Impact of cetuximab on PFS in patients with *RAS/BRAF* V600E wild-type mCRC compared with BSC in the CO.17 trial (**A**) and stratified by molecular subgroup (**B**).

EGFR therapy in this population. These findings support early incorporation of combination *BRAF*–directed treatment rather than single-agent anti-EGFR therapy (23).

Our prevalence estimate of expanded *RAS* mutations (56%) agrees with other series, where pooled estimates suggest *RAS* mutations occur in 55.9% of mCRCs (24). By combining *RAS* and *BRAF* V600E alterations, the population expected to benefit from single-agent anti-EGFR therapy drops to only 41% in our study. Interestingly, we only detected an additional 6 (2%) patients with low allele frequency mutations. This is lower than that others have reported, and may reflect the impact of tumor microdissection or utilization of a threshold for the assay associated with low rates of false positive results (1% instead of 0.1%). Improved methodologies for high-depth sequencing have been developed, although the clinical relevance of such higher sensitivity approaches remains unclear given the low prevalence of this population and difficulty confirming lack of benefit.

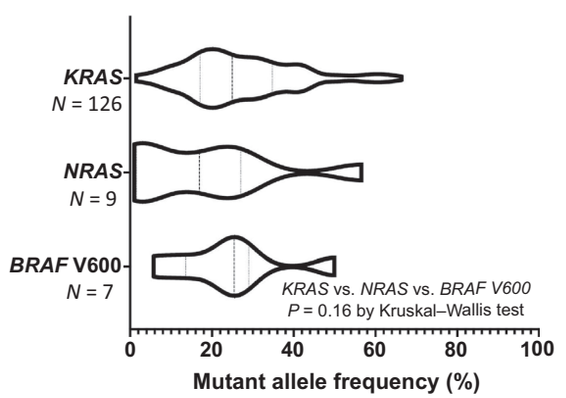

Figure 3.
Violin plot displaying the MAF distribution density of detected mutations in *KRAS, NRAS*, and *BRAF*.

In our study, three individuals had tumors harboring mutations at MAF < 5% who received anti-EGFR therapy. One of these patients had a response to cetuximab, suggesting a potential gradient of efficacy based on the MAF of mutant *RAS* in a tumor. This is supported by the CRYSTAL trial, where a gradient of activity was noted among patients based on allele frequency of *RAS* mutations (18). By using BEAMing technology, we were able to provide better stratification of patients. Not only were there 6 patients with mutations occurring between 1% and 5% MAF, but we also identified 34 patients that were *KRAS* wild-type by Sanger sequencing. This suggests there were "intermediate" allele frequency mutations that were not detected with Sanger sequencing (threshold for detection between MAF 10%–20%), however, current next-generation sequencing assays may have identified them (1, 13). Indeed, the median MAF of these 34 discordant cases

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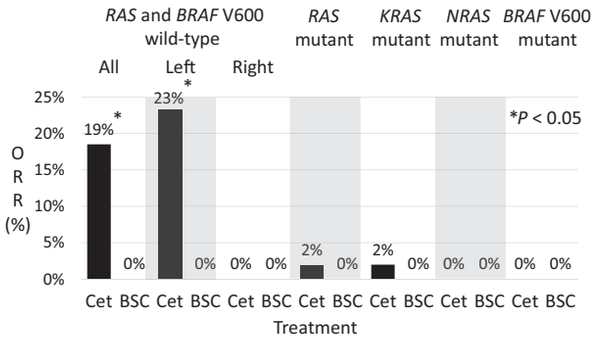

Figure 4.
ORR of patients in CO.17 trial receiving cetuximab or BSC.

was 20%. Although many of these samples should have had variants detected by Sanger's threshold, an important distinction between the original assessment of *KRAS* for CO.17 trial and this analysis is that microdissection was performed in our updated analysis. Therefore, the detected allele frequencies are likely higher than would have been noted in the original analysis, which used whole slides. Taken together, our results lend further support to the need for high sensitivity assays in the clinic.

Current guidelines suggest assays need a 5% MAF limit of detection for *RAS* mutations and our work suggests the number of additional patients identified with more sensitive assays is relatively small (12). While only 3 patients with low MAF *RAS* mutations were treated with cetuximab, 1 of these patients had a response and a PFS of 11.2 months, while 2 others with low MAF mutations had PFS of 2.7 and 3.7 months. In the CRYSTAL trial of FOLFIRI ± cetuximab, high sensitivity BEAMing identified 23 of 430 (5.3%) patients with *RAS* mutations outside of codon 12/13 occurring at allele frequencies of 0.1%–5%. In this group, the addition of anti-EGFR agents provided a signal toward benefit (HR, 0.57; 95% CI, 0.33–1.01; ref. 18). While the low allele frequency of the responding patient's mutation in CO.17 trial may explain the activity of cetuximab, the mutation was *KRAS* A59T, which has previous case reports of response and is one of these least well studied *RAS* mutations, with only 7 patients harboring this alteration in the PRIME trial that defined expanded *RAS* as a biomarker (3, 25). Taken together, both low allele frequency mutations and certain expanded *RAS* mutations are sufficiently uncommon that it is unlikely we will ever conclusively establish their role as predictive biomarkers. Hopefully, increasing use of ctDNA will provide further insights into subclonal *RAS* dynamics.

ctDNA provides great promise for detecting acquired resistance to targeted therapies and evaluating evolutionary changes in cancers. Previous work has demonstrated that *RAS*-mutant clones develop during anti-EGFR therapy (26, 27) These variants tend to have lower allele frequency than mutations present at baseline, and in Morelli and colleagues' report, 35% of them were found in primary tissue when assessed with high sensitivity BEAMing, with sensitivity beyond standard clinical tests. It remains unclear whether the utility of ctDNA may better select baseline *RAS* status compared with tissue, however, it does allow dynamic surveillance of resistance, which is unique. In mCRC, many acquired resistance mechanisms have been shown to decay over time, allowing anti-EGFR rechallenge as a treatment consideration (10, 28). The improvement in median PFS from 1.8 to 5.4 months in *RAS/BRAF* V600 wild-type patients in CO.17 trial sets a target for these rechallenge efforts. Given that many anti-EGFR rechallenge concepts include additional agents, and in the context of the ever rising costs of oncology drugs, it is essential that combinatorial strategies demonstrate clear superiority to single-agent rechallenge (9).

This study further supports the combinatorial treatment strategy for *BRAF* V600E mCRC as single-agent anti-EGFR did not improve PFS in *BRAF* V600E–mutated mCRC (23). Unfortunately, only 15 patients with *BRAF* V600 mutations were evaluable in CO.17 trial for a test of interaction, which neared significance ($P = 0.089$) despite small numbers. As CO.17 trial accrued in the treatment refractory population, it is not surprising that we saw a low prevalence of *BRAF* V600 mutations given their poor prognosis. Given the lack of benefit to date with anti-EGFR therapy, patients with *BRAF* V600E mutations should be prioritized for combinatorial strategies, which have shown significant activity in this population (23).

Despite the important findings of our study, it must be interpreted in the context of several limitations. As CO.17 trial completed enrollment over a decade ago, previous correlative analyses have exhausted much of the tissue and we could only analyze a subset of patients. Bias may be introduced into some analyses by the fact that certain patients had remaining tissue available, while others did not. However, we noted no differences in OS, PFS, or RR between the historic analysis and the updated analysis when assessing the BSC arm for prognosis in either the *RAS/BRAF*-mutant group or the wild-type group. The small number of patients who had *BRAF* or low allele frequency mutation means that findings among these groups must be interpreted in the context of the wide CI surrounding treatment effect. When the trial was planned, the importance of *RAS* was not understood and as such our analyses are *post hoc* and were not part of the original statistical plan. This is often the case with biomarker discovery, and all current evidences supporting *RAS* mutations as predictive are *post hoc*.

In conclusion, we provide updated evidence that patients with mCRC harboring expanded *RAS* or *BRAF* V600E mutations lack benefit following single-agent cetuximab. Our work demonstrates improved patient selection with the use of a high sensitivity assay that reclassified 14% of tumors as *RAS* mutated compared with Sanger sequencing. Subclonal mutations with MAF < 5% were uncommon, occurring in 2% of patients, and remain of unclear significance. We hope this updated work informs future anti-EGFR combinatorial strategies by setting a benchmark for the activity of single-agent cetuximab using a modern high sensitivity assay.

Authors' Disclosures

J.M. Loree reports grants and personal fees from Ipsen and personal fees from Taiho, Amgen, Bayer, Eisai, Pfizer, and Novartis outside the submitted work. D.L. Edelstein reports personal fees from Sysmex Inostics (employment) during the conduct of the study. H. Quinn reports personal fees from Sysmex Inostics (employment) during the conduct of the study and outside the submitted work. T. Price reports grants from Amgen outside the submitted work. J.R. Zalcberg reports grants from BMS and NHMRC during the conduct of the study and personal fees from Khloris, STA, Hubro Therapeutics, Bayer, MSD, and CEND Therapeutics outside the submitted work. C.S. Karapetis reports personal fees from Merck, Amgen, and Roche outside the submitted work. P. Waring reports other from AstraZeneca (employment), Pillar Bioscience (consultant), and Xing Technologies (consultant) outside the submitted work. S.R. Hamilton reports nonfinancial support from MD Anderson Cancer Center and Thermo Fisher Scientific during the conduct of the study, nonfinancial support from HalioDx, Illumina, ECOG-ACRIN Cancer Research Group, Loxo Oncology; grants from University of Texas and Leidos; personal fees from Fred Hutchinson Cancer Research Center, Buffett Cancer Center, Roche, Bristol Myers Squibb, Merck, and Incyte; and grants and nonfinancial support from Guardant Health outside the submitted work. S. Kopetz reports personal fees from Lutris, Navire Pharma, Symphogen, and Jacobio; grants and personal fees from Roche, EMD Serono, Boehringer

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Authors' Contributions

J.M. Loree: Conceptualization, data curation, formal analysis, visualization, methodology, writing-original draft, project administration, writing-review and editing. **A. Dowers:** Data curation, formal analysis, investigation, project administration, writing-review and editing. **D. Tu:** Resources, data curation, formal analysis, validation, investigation, methodology, writing-review and editing. **D.J. Jonker:** Resources, investigation, methodology, writing-review and editing. **D.L. Edelstein:** Resources, data curation, formal analysis, validation, investigation, methodology, writing-review and editing. **H. Quinn:** Resources, data curation, formal analysis, validation, investigation, methodology, writing-review and editing. **F. Holtrup:** Resources, data curation, formal analysis, investigation, methodology, writing-review and editing. **T. Price:** Resources, investigation, methodology, writing-review and editing. **J.R. Zalcberg:** Resources, investigation, methodology, writing-review and editing. **M.J. Moore:** Resources, investigation, methodology, writing-review and editing. **C.S. Karapetis:** Resources, investigation, methodology, writing-review and editing. **C.J. O'Callaghan:** Resources, data curation, methodology, project administration, writing-review and editing. **P. Waring:** Conceptualization, resources, data curation, formal analysis, supervision, investigation, visualization, methodology, writing-review and editing. **H.F. Kennecke:** Conceptualization, resources, supervision, investigation, methodology, writing-review and editing. **S.R. Hamilton:** Conceptualization, resources, supervision, funding acquisition, methodology, writing-review and editing. **S. Kopetz:** Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing.

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References

1. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
2. Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, Karapetis CS. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized controlled trials. Ann Oncol 2015;26:13–21.
3. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Final results from PRIME: randomized phase III study of panitumumab with FOLFOX4 for first-line treatment of metastatic colorectal cancer. Ann Oncol 2014;25:1346–55.
4. Venook AP, Niedzwiecki D, Innocenti F, Fruth B, Greene C, O'Neil BH, et al. Impact of primary tumor location on overall survival and progression free survival in patients with metastatic colorectal cancer: analysis of all RAS wt subgroup on CALGB/SWOG 80405 (Alliance). J Clin Oncol 2016;34.
5. Arnold D, Lueza B, Douillard J-Y, Peeters M, Lenz H-J, Venook A, et al. Prognostic and predictive value of primary tumour side in patients with RAS wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials. Ann Oncol 2017;28:1713–29.
6. Holch JW, Ricard I, Stintzing S, Modest DP, Heinemann V. The relevance of primary tumour location in patients with metastatic colorectal cancer: a meta-analysis of first-line clinical trials. Eur J Cancer 2017;70:87–98.
7. Boeckx N, Koukakis R, Beeck K, Kafatos G, Deschoolmeester V, Rolfo C, et al. Primary tumor sidedness impacts on prognosis and treatment outcome: results from three randomized studies of panitumumab plus chemotherapy versus chemotherapy or chemotherapy plus bevacizumab in 1st and 2nd line RAS/BRAF WT mCRC. Available from: https://www.annalsofoncology.org/article/S0923-7534(19)43721-6/fulltext.
8. Rowland A, Dias MM, Wiese MD, Kichenadasse G, McKinnon RA, Karapetis CS, et al. Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. Br J Cancer 2015;112:1888–94.
9. Parseghian CM, Napolitano S, Loree JM, Kopetz S. Mechanisms of innate and acquired resistance to anti-EGFR therapy: a review of current knowledge with a focus on rechallenge therapies. Clin Cancer Res 2019;25:6899–08.
10. Parseghian CM, Loree JM, Morris VK, Liu X, Clifton KK, Napolitano S, et al. Anti-EGFR resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. Ann Oncol 2019;30:243–9.
11. Cremolini C, Rossini D, Dell'Aquila E, Lonardi S, Conca E, Del Re M, et al. Rechallenge for patients with RAS and BRAF wild-type metastatic colorectal cancer with acquired resistance to first-line cetuximab and irinotecan. JAMA Oncol 2019;5:343–50.
12. Sepulveda AR, Hamilton SR, Allegra CJ, Grody W, Cushman-Vokoun AM, Funkhouser WK, et al. Molecular biomarkers for the evaluation of colorectal cancer: guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and the American Society of Clinical Oncology. J Clin Oncol 2017;35:1453–96.
13. Loree J, Kopetz S, Raghav K. Current companion diagnostics in advanced colorectal cancer; getting a bigger and better piece of the pie. J Gastrointest Oncol 2017;8:199–212.
14. Ciardiello F, Normanno N, Maiello E, Martinelli E, Troiani T, Pisconti S, et al. Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 2014;25:1756–61.
15. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 2006;3:551–9.
16. Tougeron D, Lecomte T, Pagès JC, Villalva C, Collin C, Ferru A, et al. Effect of low-frequency KRAS mutations on the response to anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol 2013;24:1267–73.
17. Dono M, Massucco C, Chiara S, Sonaglio C, Mora M, Truini A, et al. Low percentage of KRASMutations revealed by locked nucleic acid polymerase chain reaction: implications for treatment of metastatic colorectal cancer. Mol Med 2012;18:1519–26.
18. Van Cutsem E, Lenz H-J, Köhne C-H, Heinemann V, Tejpar S, Melezínek I, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. J Clin Oncol 2015;33:692–700.
19. Karapetis CS, Jonker D, Daneshmand M, Hanson JE, O'Callaghan CJ, Marginean C, et al. PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer-results from NCIC CTG/AGITG CO.17. Clin Cancer Res 2014;20:744–53.
20. Rago C, Huso DL, Diehl F, Karim B, Liu G, Papadopoulos N, et al. Serial assessment of human tumor burdens in mice by the analysis of circulating DNA. Cancer Res 2007;67:9364–70.
21. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci U S A 2003;100:8817–22.
22. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–47.
23. Kopetz S, Grothey A, Yaeger R, Van Cutsem E, Desai J, Yoshino T, et al. Encorafenib, binimetinib, and cetuximab in BRAF V600E–mutated colorectal cancer. N Engl J Med 2019;381:1632–43.
24. Peeters M, Kafatos G, Taylor A, Gastanaga VM, Oliner KS, Hechmati G, et al. Prevalence of RAS mutations and individual variation patterns among patients

with metastatic colorectal cancer: a pooled analysis of randomised controlled trials. Eur J Cancer 2015;51:1704–13.
25. Loree JM, Kopetz S. Why a one size fits all approach to RAS might not fit colorectal cancer. JNCCN J Natl Compr Cancer Netw 2017;15:545–47.
26. Kim TW, Peeters M, Thomas A, Gibbs P, Hool K, Zhang J, et al. Impact of emergent circulating tumor DNA Ras mutation in panitumumab-treated chemoresistant metastatic colorectal cancer. Clin Cancer Res 2018;24:5602–09.
27. Morelli MP, Overman MJ, Dasari A, Kazmi SMA, Mazard T, Vilar E, et al. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann Oncol 2015;26:731–736.
28. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486:532–6.

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Expanded Low Allele Frequency *RAS* and *BRAF* V600E Testing in Metastatic Colorectal Cancer as Predictive Biomarkers for Cetuximab in the Randomized CO.17 Trial

Jonathan M. Loree, Anthony Dowers, Dongsheng Tu, et al.

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