

Broad Detection of Alterations Predicted to Confer Lack of Benefit From EGFR Antibodies or Sensitivity to Targeted Therapy in Advanced Colorectal Cancer

ANDREW RANKIN,^a SAMUEL J. KLEMPNER,^b RACHEL ERLICH,^a JAMES X. SUN,^a AXEL GROTHEY,^c MARWAN FAKIH,^d THOMAS J. GEORGE JR.,^e JEEYUN LEE,^f JEFFREY S. ROSS,^{a,g} PHILIP J. STEPHENS,^a VINCENT A. MILLER,^a SIRAJ M. ALI,^a ALEXA B. SCHROCK^a

^aFoundation Medicine Inc., Cambridge, Massachusetts, USA; ^bThe Angeles Clinic & Research Institute, Los Angeles, California, USA; ^cMayo Clinic Cancer Center, Rochester, Minnesota, USA; ^dCity of Hope Comprehensive Cancer Center, Duarte, California, USA; ^eCollege of Medicine, University of Florida, Gainesville, Florida, USA; ^fDepartment of Hematology and Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea; ^gDepartment of Pathology and Laboratory Medicine, Albany Medical College, Albany, New York, USA

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ABSTRACT

Introduction. A *KRAS* mutation represented the first genomic biomarker to predict lack of benefit from anti-epidermal growth factor receptor (EGFR) antibody therapy in advanced colorectal cancer (CRC). Expanded *RAS* testing has further refined the treatment approach, but understanding of genomic alterations underlying primary and acquired resistance is limited and further study is needed.

Materials and Methods. We prospectively analyzed 4,422 clinical samples from patients with advanced CRC, using hybrid-capture based comprehensive genomic profiling (CGP) at the request of the individual treating physicians. Comparison with prior molecular testing results, when available, was performed to assess concordance.

Results. We identified a *RAS/RAF* pathway mutation or amplification in 62% of cases, including samples harboring *KRAS* mutations outside of the codon 12/13 hotspot region in 6.4% of cases. Among cases with *KRAS* non-codon 12/13

alterations for which prior test results were available, 79 of 90 (88%) were not identified by focused testing. Of 1,644 *RAS/RAF* wild-type cases analyzed by CGP, 31% harbored a genomic alteration (GA) associated with resistance to anti-EGFR therapy in advanced CRC including mutations in *PIK3CA*, *PTEN*, *EGFR*, and *ERBB2*. We also identified other targetable GA, including novel kinase fusions, receptor tyrosine kinase amplification, activating point mutations, as well as microsatellite instability.

Conclusion. Extended genomic profiling reliably detects alterations associated with lack of benefit to anti-EGFR therapy in advanced CRC, while simultaneously identifying alterations potentially important in guiding treatment. The use of CGP during the course of clinical care allows for the refined selection of appropriate targeted therapies and clinical trials, increasing the chance of clinical benefit and avoiding therapeutic futility. *The Oncologist* 2016;21:1306–1314

Implications for Practice: Comprehensive genomic profiling (CGP) detects diverse genomic alterations associated with lack of benefit to anti-epidermal growth factor receptor therapy in advanced colorectal cancer (CRC), as well as targetable alterations in many other genes. This includes detection of a broad spectrum of activating *KRAS* alterations frequently missed by focused molecular hotspot testing, as well as other *RAS/RAF* pathway alterations, mutations shown to disrupt antibody binding, RTK activating point mutations, amplifications, and rearrangements, and activating alterations in downstream effectors including PI3K and MEK1. The use of CGP in clinical practice is critical to guide appropriate selection of targeted therapies for patients with advanced CRC.

INTRODUCTION

Colorectal adenocarcinoma (CRC) is the third most common cancer in men and second in women, accounting for an estimated 694,000 deaths annually worldwide [1]. Despite screening efforts, nearly 20% of patients present with metastatic disease, which is

associated with a poor prognosis and median overall survival of 24–30 months [2–5]. The monoclonal antibodies directed against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab, have improved outcomes in patients without

Correspondence: Alexa B. Schrock, Ph.D., Foundation Medicine, Inc., 150 Second Street, Second Floor, Cambridge, Massachusetts 02141, USA. Telephone: 617-418-2200; E-Mail: aschrock@Foundationmedicine.com Received April 10, 2016; accepted for publication June 20, 2016; published Online First on September 28, 2016. ©AlphaMed Press 1083-7159/2016/\$20.00/0 <http://dx.doi.org/10.1634/theoncologist.2016-0148>

oncogenic alterations in *RAS*, and multiple studies have shown that *KRAS* mutations, as well as *NRAS* mutations and *BRAF* V600E, are associated with lack of clinical benefit from anti-EGFR therapies [6–11]. However, *RAS* and *RAF* mutations only account for lack of response to anti-EGFR therapies in up to 60% of CRC patients [12], suggesting that a significant fraction of *RAS/RAF* wild-type patients do not respond and additional predictors of response remain to be established.

Activating mutations in *KRAS* have been reported in approximately 40% of CRC, and point mutations at codons 12 or 13 of exon 2 account for 80%–90% of these alterations [12, 13]. However, additional mutations have been reported in exons 2, 3, and 4, and have also been shown to predict resistance to cetuximab and panitumumab [14–16]. Current guidelines now endorse extended *RAS* testing (exons 2–4 of *KRAS* and *NRAS*) to reflect the evidence that anti-EGFR therapy should be avoided in *RAS*-altered advanced CRC [17]. Smaller studies have suggested that *RAS* amplification and alterations in genes, including *PIK3CA*, *EGFR*, and *ERBB2*, are associated with resistance to cetuximab or panitumumab in CRC, but a large comprehensive study reporting the incidence and diversity of these alterations is lacking [18–21]. Furthermore, assessment of the genomic landscape of CRC has used mainly treatment-naïve, largely nonmetastatic primary tumors or focused assessments of specimens from patients with advanced disease [22, 23]. We present the genomic profiles and clinical implications of 4,422 CRC cases assayed in the course of clinical care, often in the setting of metastatic disease.

MATERIALS AND METHODS

A series of 4,422 CRC cases was prospectively collected and assayed with a validated, hybrid-capture-based comprehensive genomic profiling (CGP) assay between August 2012 and June 2015. DNA was extracted from 40 microns of formalin-fixed, paraffin-embedded sections, and CGP was performed on hybridization-captured, adaptor-ligation-based libraries to a mean coverage depth of $>650\times$ for at least 236 cancer-related genes plus 47 introns from 19 genes frequently rearranged in cancer, as described previously [24]. Full exons for all included genes were sequenced. All classes of genomic alterations (GAs) were identified, including base-pair substitutions, insertions and deletions, copy number alterations, and rearrangements. Focal amplifications are called at segments with ≥ 6 copies (or ≥ 7 for triploid; ≥ 8 for tetraploid tumors) in samples with purity of $>20\%$. Microsatellite instable (MSI-H) or stable (MSS) status as a measure of mismatch repair deficiency was determined using a proprietary computational algorithm. Tumors were classified as MSI-H or MSS using a principal component 1 cutoff value of < -8.5 or > -4 , respectively. Values > -8.5 and < -4 were classified as MSI-ambiguous [25]. Results from prior *KRAS* molecular testing were obtained by review of available medical records provided by the treating physician. Ordinal relationships were examined using the Wilcoxon-Mann-Whitney test; the relationship between the presence of *ERBB2* alterations and *RAS/RAF* status was examined using a chi-square test. Approval for this study, including a waiver of informed consent and a Health Insurance Portability and

Accountability Act waiver of authorization, was obtained from the Western Institutional Review Board (Protocol no. 20152817).

RESULTS

Detection of *KRAS* Mutations by CGP

CGP identified 51% of cases (2,251 of 4,422) as positive for any *KRAS* mutation, including 284 cases (6.4% of total CRC cases) with activating *KRAS* mutations occurring outside of codons 12 and 13 (Table 1). Non-codon 12/13 *KRAS* alterations consisted of mutations at A146 ($n = 137$; 48%) and Q61 ($n = 94$; 33%), as well as 8 other positions at which mutations have been shown to be activating and oncogenic (Fig. 1). Eight CRC cases otherwise negative for *RAS* or *BRAF* alterations were also found to harbor *KRAS* insertions in the codon 12/13 hotspot region.

We examined provided pathology reports for CRC cases with *KRAS* non-12/13 activating mutations detected by CGP ($n = 284$), and prior focused *KRAS* molecular testing results were available for 90 cases. Of these, 79 (88%) reported a *KRAS* wild-type result, indicating that the *KRAS* non-12/13 alteration was not detected by hotspot testing in the vast majority of cases. This represents a missed clinical benefit rate of 98% (49 of 50) for patients with A146 mutations, 67% (16 of 24) for patients with Q61 mutations, and 74% (25 of 34) for activating point mutations at 9 other codons detected by CGP (Fig. 1). *KRAS* insertions in the 12/13 hotspot region were missed in 5 of 6 cases (83%) by prior hotspot testing (Fig. 1).

Non-*KRAS* Ras/Raf Family Alterations

In addition to *KRAS* mutations, *NRAS* and *HRAS* mutations were found in 161 (3.6%) and 7 (0.2%) of all CRC cases analyzed, respectively (Table 1). *RAS* amplification was observed in 86 CRC cases (1.9%), including 75 cases with *KRAS* amplification. *BRAF* mutations were observed in 321 (7.3%) CRC cases and included V600 mutations ($n = 233$; 72.6%), non-V600 activating mutations ($n = 34$; 10.6%), and inactivating mutations known to lead to feedback activating of *RAF/MEK* signaling ($n = 54$; 16.8%) [26]. All mutations at *BRAF* position 600 were V600E, except for one case with V600G. In addition, activating *BRAF* rearrangements were observed in seven cases, including three with *TRIM24-BRAF* fusions (Table 1).

RAF and *RAS* alterations were not mutually exclusive in our dataset, with 47 cases harboring 2 or more *RAS* mutations (Fig. 2A). *BRAF* inactivating mutations co-occurred with *RAS* mutations in 22 of 54 cases (41%) and *BRAF* rearrangements co-occurred with *RAS* mutations in 2 of 7 cases (29%); however, *BRAF* V600 mutations were the sole *RAS/RAF* alteration in 228 of 233 cases (98%). *RAS* amplification co-occurred with other *RAS/RAF* alterations in 31 of 86 cases (36%) (Fig. 2A), and *BRAF* amplification was observed in 4 total cases, but co-occurred with a *KRAS* or *BRAF* mutation in 3 of 4 cases (data not shown).

RTK Alterations

We observed RTK mutations in 96 cases (2%) including EGFR extracellular (EC) domain mutations ($n = 10$), and *ERBB2* EC

Table 1. Incidence of RAS/RAF alterations and other alterations predicted to negatively affect response to anti-EGFR therapy in CRC

Alteration	CRC cases, n (%) n = 4,422	RAS WT CRC cases, n (%) n = 1,956	RAS/RAF WT cases, n (%) n = 1,664
RAS/RAF alteration	2,758 (62)	N/A	N/A
Mutation			
KRAS	2,251 (51)	N/A	N/A
NRAS	161 (4)	N/A	N/A
HRAS	7 (0.2)	N/A	N/A
ARAF	3 (0.1)	2 (0.1)	N/A
BRAF	321 (7)	282 (14)	N/A
Amplification			
KRAS	75 (2)	N/A	N/A
NRAS	6 (0.1)	N/A	N/A
HRAS	5 (0.1)	N/A	N/A
ARAF	3 (0.1)	2 (0.1)	N/A
BRAF	4 (0.1)	2 (0.1)	N/A
BRAF rearrangement (activating)	7 (0.2)	5 (0.3)	N/A
RTK mutation	96 (2)	57 (3)	53 (3)
EGFR EC domain	10 (0.2)	7 (0.4)	6 (0.4)
ERBB2 EC domain	15 (0.3)	10 (0.5)	9 (0.5)
ERBB2 KD	69 (2)	37 (2)	35 (2)
ERBB2 truncation (activating)	4 (0.1)	3 (0.2)	3 (0.2)
RTK amplification	480 (11)	271 (14)	260 (16)
ERBB2	137 (3)	115 (6)	114 (7)
MET	39 (1)	26 (1)	26 (2)
FLT3	320 (7)	143 (7)	133 (8)
RTK rearrangement (activating)	16 (0.4)	14 (1)	14 (1)
ALK	3 (0.1)	2 (0.1)	2 (0.1)
RET	8 (0.2)	8 (0.4)	8 (0.5)
FGFR1	2 (0.1)	2 (0.1)	2 (0.1)
FGFR3	1 (0.02)	0	0
NTRK1	2 (0.1)	2 (0.1)	2 (0.1)
MEK1 mutation	41 (1)	35 (2)	31 (2)
PIK3CA mutation	746 (17)	209 (11)	150 (9)
PTEN deletion or truncation	289 (7)	113 (6)	73 (4)
No alterations in any of the above	1,157 (26)	1,157 (59)	1,157 (70)

Abbreviations: CRC, colorectal cancer; EC, extracellular domain; KD, kinase domain; WT, wild-type.

domain ($n = 15$) and kinase domain (KD; $n = 69$) mutations and activating truncations ($n = 4$) (Table 1). These included eight cases with EGFR G456R or S492R mutations. We also identified one case with an EGFR L858R activating kinase domain mutation, but did not observe EGFR exon 19 deletion or exon 20 insertion alterations. The most common ERBB2 mutations were S310F/Y ($n = 13$), R678Q ($n = 18$), and V842I ($n = 18$). Twelve cases with ERBB2 point mutations also had concurrent ERBB2 amplification. We observed amplification of FLT3, ERBB2, and MET in 7%, 3%, and 1% of cases, respectively. Activating RTK rearrangements involving RET ($n = 8$), ALK ($n = 3$), FGFR1 ($n = 2$), NTRK1 ($n = 2$), and FGFR3 ($n = 1$) were detected in 16 cases (0.4%), including

a novel CENPF-ALK fusion. RET fusion partners included NCOA4 ($n = 4$), CCDC6 ($n = 3$), and TRIM24 ($n = 1$).

MEK1, PTEN, and PI3K Alterations

We identified MEK1 (MAP2K1) mutations in 41 cases (0.9%), PTEN homozygous deletion or inactivating truncation in 289 cases (7%), and mutations in the catalytic subunit of PI3K (PIK3CA) in 748 cases (17%) (Table 1). MEK1 mutations included K57E/N/T ($n = 19$), Q56P ($n = 6$), E203K ($n = 6$), C121S ($n = 4$), and other ($n = 6$). MEK1 mutations co-occurred with BRAF mutations in 4 of 41 cases, including 1 case with BRAF K601N, but did not co-occur specifically with BRAF V600 alterations. Of cases with PTEN-inactivating alterations, homozygous deletion and truncation were relatively equally

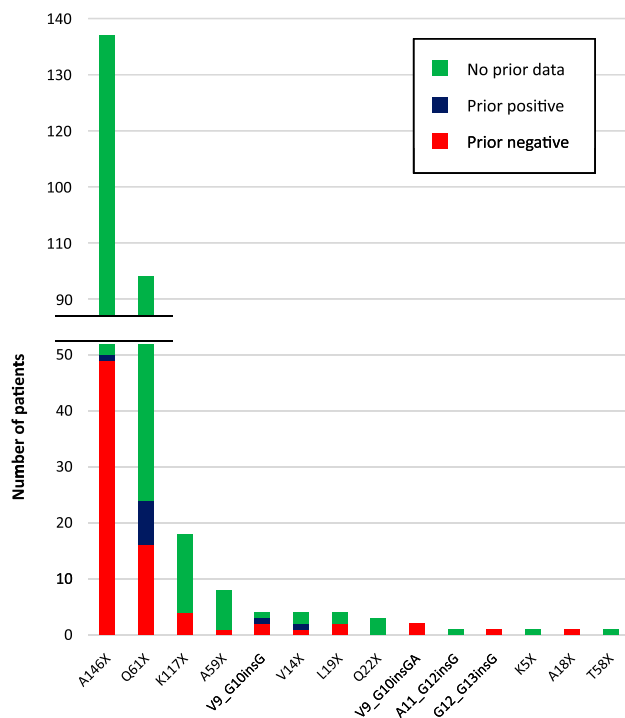


Figure 1. Missed detection of KRAS non-G12/13 mutations by prior focused molecular testing. KRAS mutations detected by comprehensive genomic profiling and missed by prior focused molecular testing (red bars), detected by prior focused molecular testing (blue bars), or with no record of prior molecular testing in provided pathology report (green bars).

represented (48% and 52% of cases, respectively). The majority of *PIK3CA* mutations were activating and occurred most commonly in exons 9 and 20, specifically at E545 (244), E542 (130), and H1047R (134).

Co-occurrence With RAS/RAF Alterations

RTK alterations were not mutually exclusive with *RAS/RAF* alterations; most notably, *FLT3* amplification co-occurred with *KRAS* point mutations in 48% of cases (152 of 320) and with *RAS/RAF* alterations in general in 58% of cases (187 of 320). *MET* amplification and *ERBB2* amplification were less likely to co-occur with *RAS/RAF* alterations, and tumors harboring amplification of these receptors were extended *RAS/RAF* wild-type in 26 of 39 cases (67%) and 114 of 137 cases (83%), respectively (Fig. 3). Interestingly, *FLT3* and *MET* amplification were significantly higher in *RAS/RAF* wild-type cases (mean copy number: *FLT3*, 18 copies; *MET*, 34 copies) compared with cases in which a *RAS* or *RAF* alteration co-occurred (mean copy number: *FLT3*, 12 copies; *MET*: 9 copies; $p < .003$ and $p < .001$, respectively); whereas *ERBB2* amplification was consistently high regardless of *RAS/RAF* status (mean *ERBB2* copy number: 60 copies in *RAS/RAF* wild-type cases, and 64 copies in *RAS/RAF* positive cases; $p = .300$).

Tumors with RTK point mutations also harbored concurrent *RAS/RAF* alterations in a significant fraction of cases. Mutations in the EC domain of EGFR and *ERBB2* were observed in the presence of co-occurring *KRAS* mutations in 4 of 10 cases (40%) and 6 of 15 cases (40%), respectively. Half the cases with *ERBB2* KD mutations harbored concurrent *RAS/RAF* mutations (35 of 69 cases; 51%) (Fig. 3), and in 28 of 35 of these *RAS/RAF*

positive cases, the concurrent alteration was an activating *KRAS* mutation at G12 or 13 (data not shown). Notably, cases with activating *ERBB2* truncations or RTK rearrangements lacked co-occurring *RAS/RAF* mutations in 3 of 4 cases (75%) and 14 of 16 cases (88%) (Fig. 3).

The majority of tumors with MEK1 mutations (31 of 41; 76%) did not harbor co-occurring *RAS/RAF* alterations. However, *PIK3CA* mutations co-occurred with *RAS/RAF* alterations in 80% of cases (596 of 746), including 509 of 746 cases (68%) with concurrent *KRAS* mutations (Fig. 3), and also co-occurred with other potential driver alterations (Fig. 2B). *PTEN* inactivating alterations also co-occurred with *RAF/RAF* alterations in 75% of cases (216 of 289) (Fig. 3).

Microsatellite Instability

MSI data were available for a subset of patients with advanced CRC in this series. Overall, 4.0% of tumors (51 of 1,268) were MSI-H, and 96% of cases were MSS. Similarly, 4.2% of *RAS/RAF* wild-type cases were MSI-H. However, further analysis of alteration subclasses revealed that BRAF V600 mutated tumors were MSI-H in 21.5% of cases (14 of 65), and microsatellite instability was also enriched to a lesser degree in tumors with MEK1, *ERBB2* KD, and PI3K mutations (Table 2). Interestingly, 30% of tumors with *PTEN* truncation were MSI-H, compared with only 2% of tumors with *PTEN* homozygous deletion. Tumors with *KRAS* mutations, regardless of whether the mutation affected the codon 12/13 hotspot region, were less likely overall to harbor microsatellite instability (MSI-H in 2.5% of cases). In this series, tumors with available data harboring other activating alterations, including *NRAS* mutations ($n = 46$), BRAF non-V600 mutations ($n = 36$), *RAS* amplification ($n = 24$), *ERBB2* amplification ($n = 46$), *MET* amplification ($n = 10$), and *FLT3* amplification ($n = 93$), were exclusively MSS (Table 2).

DISCUSSION

Using CGP, we set out to characterize the genomic landscape of advanced CRC in clinical practice with a focus on coexisting alterations that may underlie the poor response to EGFR-directed therapies in patients with reportedly *KRAS* wild-type tumors, and to identify other clinically relevant genomic alterations. Largely consistent with prior reports, we identified *KRAS* mutations in 51% of cases (2,251 of 4,422) and other *RAS/RAF* alterations in an additional 507 cases. *KRAS* amplification was found in 75 cases (1.7%)—slightly higher than in a previous study that showed amplification as well as mutation was associated with resistance to cetuximab and panitumumab [27]. Importantly, our series differs from prior analyses in both the size of the data set (4,422 vs. 276 in the Cancer Genome Atlas) and use of samples from advanced disease more reflective of the population considered for EGFR-directed therapies [22]. Although EGFR protein expression detected by immunohistochemistry was the original biomarker used to guide selection of anti-EGFR monoclonal antibody therapies in CRC, this test is no longer clinically relevant and was not evaluated in the current study [28]. A limitation of this study is the lack of interpretable clinical outcomes in patients with these alterations who were treated with targeted therapy. However, for patients treated with EGFR monoclonal antibodies, these therapies

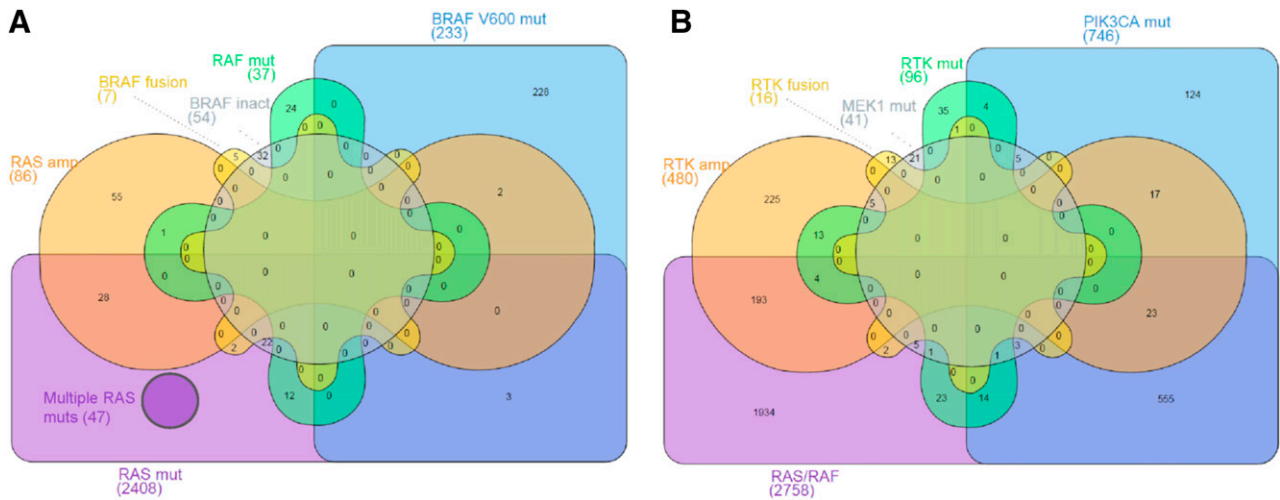


Figure 2. Overlap of RAS/RAF alterations and other potential drivers in colorectal cancer. Diagram represents overlap of colorectal patient cases with the indicated classes of genomic alterations detected by comprehensive genomic profiling. **(A):** Overlap of cases with subtypes of RAS/RAF alterations. **(B):** Overlap of all cases with RAF/RAS alterations with cases with subtypes of activating alterations in other potential drivers.

Abbreviations: amp, amplification; mut, mutation; RTK, receptor tyrosine kinase.

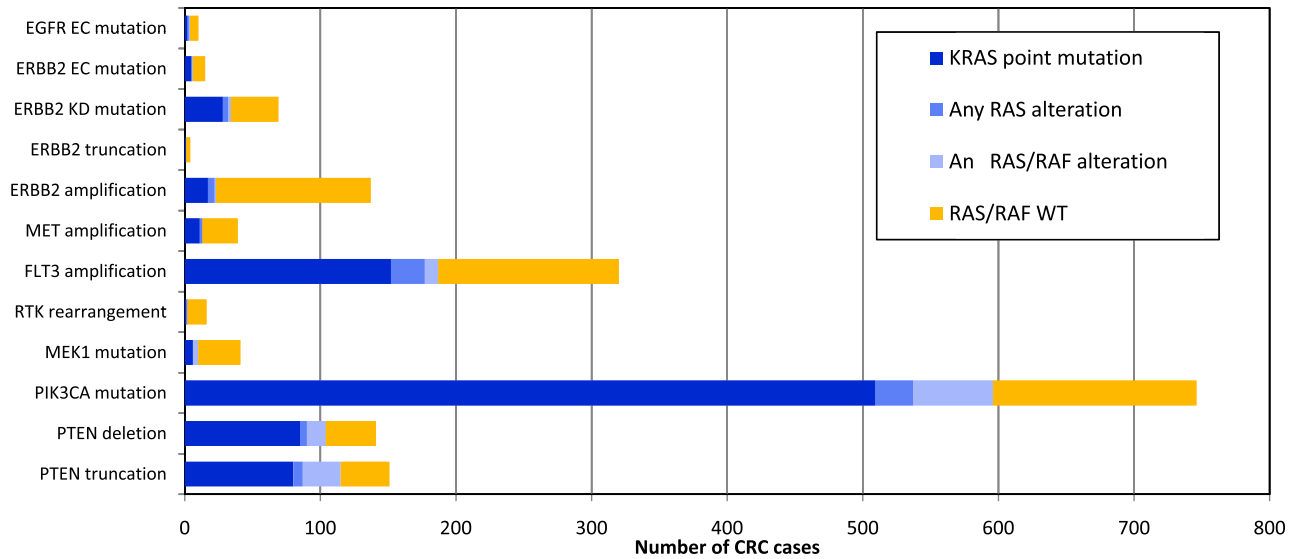


Figure 3. Quantitation of CRC cases with potential driver alterations and co-occurrence of *KRAS* mutations. Breakdown of specific subtypes of RTK, MEK1, *PIK3CA*, and *PTEN* alteration classes and overlap with RAS/RAF alterations. Dark blue bars specify the subset of cases that overlap specifically with *KRAS* point mutations.

Abbreviations: CRC, colorectal cancer; EC, extracellular domain; KD, kinase domain; RTK, receptor tyrosine kinase.

were given in combination with various chemotherapy backbones in the majority of cases identified, making clear assessment of the effect of EGFR inhibitor alone difficult to interpret. Furthermore, access to and treatment with targeted therapies including kinase inhibitors is still relatively uncommon in clinical practice for CRC and other gastrointestinal malignancies, making identification of these cases onerous, particularly as a number of these patients are currently enrolled in active clinical trials. Future studies focused on genomic analysis of patients treated with anti-EGFR monotherapy, as well as continued efforts to identify and assess CRC patients treated with other targeted therapies, are needed.

Using CGP, we identified nearly 1 in 3 patients with RAS/RAF wild-type tumors (507 of 1,644) that harbored at least 1 concurrent alteration that could potentially mediate resistance to an EGFR therapeutic antibody. Furthermore, in patients negative for alterations commonly detected by hotspot tests, including *KRAS* codons 12/13 and BRAF V600 mutations, CGP identified more than 1 in 2 patients (1,159 of 2,224) whose tumors harbored at least 1 concurrent alteration that could potentially mediate EGFR antibody resistance. In a significant portion, the additional alteration was itself a validated alternative therapeutic target in CRC. Interestingly, the response rates to combination chemotherapy and anti-EGFR therapy from phase III trials of *KRAS* wild-type (limited *KRAS* testing) only

Table 2. MSI in colorectal cases categorized by genomic alteration

Alteration	MSI-H	MSS	MSI-ambiguous	Total no. of cases with data	MSI-H, %
ERBB2 truncation	1	0	0	1	100.0
HRAS mut	2	1	0	3	66.7
EGFR EC	2	1	0	3	66.7
RTK rearrangement	3	3	0	6	50.0
PTEN truncation	16	37	0	53	30.2
BRAF V600 mut	14	51	0	65	21.5
MEK1 mut	2	14	0	16	12.5
ERBB2 KD	2	15	1	18	11.1
PIK3CA mut	14	187	1	202	6.9
KRAS non-G12/13 mut	2	70	0	72	2.8
KRAS G12/13 mut	14	540	0	554	2.5
PTEN deletion	1	43	0	44	2.3
NRAS mut	0	46	0	46	0.0
ARAF mut	0	1	0	1	0.0
BRAF non-V600 mut	0	36	0	36	0.0
KRAS amp	0	20	0	20	0.0
NRAS amp	0	3	0	3	0.0
HRAS amp	0	1	0	1	0.0
ARAF amp	0	1	0	1	0.0
BRAF rearrangement	0	1	0	1	0.0
ERBB2 EC	0	2	0	2	0.0
ERBB2 amp	0	46	0	46	0.0
MET amp	0	10	0	10	0.0
FLT3 amp	0	93	0	93	0.0
RAS/RAF WT	21	477	1	499	4.2
All cases	51	1,216	1	1,268	4.0

Abbreviations: amp: amplification; EC, extracellular domain; EGFR, epidermal growth factor receptor; KD, kinase domain; MSI, microsatellite instability; MSI-H, microsatellite instability high; MSS, microsatellite stable; mut, mutation; WT, wild-type.

approach 60%, indirectly suggesting that our observations may partly underlie the clinical observation [12]. Potential cetuximab/panitumumab resistance alterations in *RAS/RAF* wild-type tumors included alterations that impact antibody binding, bypass track activation, and alteration of downstream signaling cascade nodes—all well-established resistance mechanisms in cancer. Specifically, we observed EGFR EC domain mutations, which have been shown to disrupt antibody binding, and amplification of *MET* and *FLT3*, all of which have been associated with resistance to anti-EGFR therapy in CRC [19, 29–32]. Concurrent or compensatory *ERBB2* amplification is a poor prognostic indicator independent of *KRAS* and *ERBB2* mutations, and mediates resistance in a portion of anti-EGFR-treated patients [33, 34]. We observed a 5% rate ($n = 219$) of *ERBB2* mutation or amplification, which was enriched in *RAS/RAF* wild-type CRC ($n = 148$; 9%; $p < .001$).

Activating alterations downstream of RTKs are well-established resistance mechanisms to RTK-targeted therapies and we identified activating mutations in *PIK3CA* and *MAP2K1* (MEK1) and inactivating alterations in *PTEN*. MEK1 mutations have been identified in CRC patients with primary resistance to panitumumab, as well as acquired resistance to BRAF-directed therapy [19, 35]. PI3K pathway alterations,

including *PIK3CA* mutation and *PTEN* mutation and loss of expression, have been associated with poor overall response rate and shorter overall survival in patients with *KRAS* wild-type CRC treated with cetuximab and panitumumab [36–38]. However, *PIK3CA* mutation has been reported in both pre- and post-treatment biopsy specimens from CRC patients who have developed resistance to anti-EGFR therapies, and we and others observed high co-occurrence with other alterations, including *RAS* mutations, suggesting that the functional significance of *PIK3CA* mutation in CRC is less clear [21, 29]. The frequencies of RTK and MEK1 alterations reported here were similar to those observed in a recent smaller study, which suggested a poor response rate to anti-EGFR antibodies in patients with *RAS* wild-type CRC with these alterations [39]; however, we did identify some co-occurrence of MEK1 and *RAS/RAF* mutations.

To approximate the fraction of cases tested using CGP that were likely tested in the setting of acquired resistance to targeted therapy, we assessed whether the tumor biopsy specimen submitted for CGP was taken from a primary site (colon or rectum) or from a metastatic site (most commonly liver or lung). For cases with available data, 46% (2,009 of 4,343) were metastatic (supplemental

online Table 1). Unsurprisingly, tumors with EGFR EC domain mutations known to disrupt EGFR antibody binding were found in metastatic biopsy specimens in 8 of 10 cases. Generally, *RAS* and *RAF* amplification, as well as *ERBB2* EC domain mutation and *MEK1* mutation, were also associated with metastatic tumors. Notably, *ERBB2* KD mutations and *BRAF* V600 mutations were more commonly found in primary tumors of the colon or rectum (supplemental online Table 1). These observations may indicate that particular genomic alterations are more likely to occur pretreatment as primary drivers in CRC, whereas others arise post-treatment; however, further analysis is warranted.

In addition to predicting lack of sensitivity to EGFR-targeted therapies, alterations in several assayed genes have been associated with sensitivity to alternate targeted therapies. *ERBB2* amplification and mutation have emerged as targetable alterations in CRC (5% in our series) and two trials targeting *ERBB2* are ongoing in this molecularly defined group (i.e., NCT01960023 and NCT01862003 [http://www.clinicaltrials.gov]) [32, 40, 41]. Response to the multikinase inhibitor sorafenib was also observed in a CRC patient with *FLT3* amplification [42]. Furthermore, a recent study reported a response to panitumumab in combination with trametinib in a patient with a *MEK1* K57T mutation [43]. A subset of colorectal tumors with neuroendocrine features and *BRAF* V600E mutations is also predicted to respond to combined inhibition with *BRAF* and *MEK* inhibitors [44]. Although EGFR EC mutations can disrupt cetuximab and panitumumab binding, newer generations of therapeutic antibodies have shown promise in overcoming this resistance and may be effective in CRC patients with these alterations [45]. Although less common (0.4% overall), chromosomal rearrangements involving *ALK*, *RET*, and *NTRK1* in CRC are emerging as sensitive to appropriate targeted therapies [46–48]. Interestingly, rearrangements were mutually exclusive from *RAS/RAF* alterations in 88% of cases (14 of 16), possibly suggesting an oncogene-driven, biologically distinct subset of CRC requiring further investigation.

Patients with mismatch-repair-deficient CRC have been shown to have significantly improved response rates to antiprogrammed death 1 (PD-1) immune checkpoint inhibitor therapy compared with patients with mismatch repair-proficient tumors [49]. Herein, we found that 4.0% of CRC cases for which data were available, including a similar fraction of *RAS/RAF* wild-type tumors, were mismatch-repair deficient. This is lower than the 15% MSI-H level consistently reported in the literature; however, cases submitted for CGP are often metastatic, and also are most likely biased toward those determined to be MSS by prior testing and thus have not been identified as candidates for immunotherapy [50]. Interestingly, tumors with *BRAF* V600 mutations or *PTEN* truncation appeared to be enriched for mismatch-repair deficiency, suggesting that both PD-1/PD-L1 and *BRAF/MEK*- or *PI3K/AKT*-targeted therapies, respectively, or possibly a combination of immunotherapy and kinase-targeted therapy, could be effective in these patients. In contrast, mismatch-repair deficiency was not detected in tumors with other potential driver alterations, including *ERBB2*, *MET*, and *KRAS* amplification. A recent report has described a similar method from MSI testing arising from next-generation sequencing [51].

Among all CRC cases analyzed, we identified non-codon 12/13 *KRAS* mutations in 284 (6.4%). Multiple studies have shown that alterations across *KRAS* exons 2, 3, and 4

similarly predict cetuximab and panitumumab resistance, and the National Comprehensive Cancer Network (NCCN) and American Society for Clinical Oncology (ASCO) guidelines have expanded to recommend extended *KRAS* testing, as well as testing for *NRAS* and *BRAF* mutations [6, 9, 15, 16, 52]. Additionally, *KRAS* insertions within or adjacent to hotspot regions have been reported; these mutations have been characterized as activating and oncogenic, and may similarly predict resistance, although this has not yet been established [53–56].

There is no uniform standard for *KRAS* testing and the majority of commonly used, commercially available assays assess only point mutations at codons 12 and 13, and lack sufficient coverage to detect less frequent *KRAS* alterations, which have been shown to predict resistance to anti-EGFR therapy [57, 58]. Molecular test results returned to treating physicians can be misleading because published validation for the majority of assays indicating performance limitations is not readily available. Unfortunately, use of standard *KRAS* testing without understanding the limitations can lead to incorrect reporting of false-negative results for patients harboring activating *KRAS* alterations, including point mutations at Q61 and A146, as well as less common activating point mutations and insertions. We report here that 88% of *KRAS* non-12/13 mutations detected by CGP (79 of 90) were missed by prior hotspot testing. Given the established predictive value of these alterations and the expansion of current NCCN and ASCO guidelines, addressing the limitations of *KRAS* testing methods currently used in clinical practice is essential.

CONCLUSION

Beyond standardizing expanded *RAS* testing methodologies, interrogating tumors for *RAS* amplification, EGFR EC mutations, and targetable driver alterations in other genes, including *ERBB2* amplification and activating point mutations, *MET* amplification, and RTK rearrangements, should also be considered because these alterations occur in a significant fraction of patients with *RAS/RAF* wild-type CRC. CGP in the course of clinical care provides sensitive detection of all classes of *RAS/RAF*, RTK, *PI3K/AKT*, and *MEK/ERK* pathway alterations and should be used at a minimum for CRC patients who test negative for *KRAS* or *BRAF* mutations using limited panels, to detect alterations predicting both lack of response to EGFR antibodies as well as response to other available targeted therapies. Moreover, given the co-occurrence of canonical *KRAS* mutations with other oncogenic drivers, CGP may also reveal targetable alterations in *KRAS* mutant CRC. Thus, CGP could provide benefit when used as first-line testing for metastatic CRC. With the complexities of cancer genomics and the expansion of targeted compounds and immunotherapy, we anticipate CGP to play an increasing role in personalizing the treatment of advanced CRC.

AUTHOR CONTRIBUTIONS

Conception/Design: Andrew Rankin, Samuel J. Klempner, Philip J. Stephens, Vincent A. Miller, Siraj M. Ali, Alexa B. Schrock

Collection and/or assembly of data: Andrew Rankin, James X. Sun, Alexa B. Schrock

Data analysis and interpretation: Andrew Rankin, Samuel J. Klempner, Rachel Erlich, James X. Sun, Axel Grothey, Marwan Fakih, Thomas J. George Jr., Jeeyun Lee, Jeffrey S. Ross, Philip J. Stephens, Vincent A. Miller, Siraj M. Ali, Alexa B. Schrock

Manuscript writing: Andrew Rankin, Thomas J. George Jr., Alexa B. Schrock

Final approval of manuscript: Andrew Rankin, Samuel J. Klempner, Rachel Erlich, James X. Sun, Axel Grothey, Marwan Fakih, Thomas J. George Jr., Jeeyun Lee, Jeffrey S. Ross, Philip J. Stephens, Vincent A. Miller, Siraj M. Ali, Alexa B. Schrock

DISCLOSURES

Andrew Rankin: Foundation Medicine (E, OI); **Samuel J. Klempner:** Foundation Medicine (H); **Rachel Erlich:** Foundation Medicine (E, OI); **James X. Sun:** Foundation Medicine (E, OI); **Axel Grothey:** Genentech, Bayer, Eisai, Boehringer Ingelheim, Boston Biomedical

(RF); **Marwan Fakih:** Sirtex, Amgen (H), Novartis, Amgen (RF); **Thomas J. George Jr.:** NewLink Genetics, Bayer (C/A), Bayer, Bristol-Myers Squibb, NewLink Genetics, Eli Lilly (RF); **Jeffrey S. Ross:** Foundation Medicine (RF, E, OI); **Philip J. Stephens:** Foundation Medicine (E, OI); **Vincent A. Miller:** Foundation Medicine (E, OI); **Siraj M. Ali:** Foundation Medicine (E, OI, IP); **Alexa B. Schrock:** Foundation Medicine (E, OI). The other author indicated no financial relationships.

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For Further Reading:

Roberto Moretto, Chiara Cremolini, Daniele Rossini et al. Location of Primary Tumor and Benefit From Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies in Patients With RAS and BRAF Wild-Type Metastatic Colorectal Cancer. *The Oncologist* 2016;21:988–994.

Implications for Practice:

Right- and left-sided colorectal tumors have peculiar epidemiological and clinicopathological characteristics, distinct gene expression profiles and genetic alterations, and different prognoses. This study assessed the potential predictive impact of primary tumor site with regard to anti-epidermal growth factor receptor (EGFR) monoclonal antibody treatment in patients with RAS and BRAF wild-type metastatic colorectal cancer. The results demonstrated the lack of activity of anti-EGFRs in RAS and BRAF wild-type, right-sided tumors, thus suggesting a potential role for primary tumor location in driving treatment choices.