original reports

Identification of Actionable Fusions as an Anti-EGFR Resistance Mechanism Using a Circulating Tumor DNA Assay

Katherine Clifton, MD[1]; Thereasa A. Rich, MS[2]; Christine Parseghian, MD[1]; Victoria M. Raymond, MS[2]; Arvind Dasari, MD[1]; Allan Andresson Lima Pereira, MD, PhD[1]; Jason Willis, MD[1]; Jonathan M. Loree, MD[3]; Todd M. Bauer, MD[4]; Young Kwang Chae, MD, MPH, MBA[5]; Gary Sherrill, MD[6]; Paul Fanta, MD, MS[7]; Axel Grothey, MD[8]; Andrew Hendifar, MD, MPH[9]; David Henry, MD[10]; Daruka Mahadevan, MD, PhD[11]; Mohammad Amin Nezami, MD[12]; Benjamin Tan, MD[13]; Zev A. Wainberg, MD[14]; Richard Lanman, MD[2]; Scott Kopetz, MD, PhD[1]; and Van Morris, MD[1]

abstract

PURPOSE Gene fusions are established oncogenic drivers and emerging therapeutic targets in advanced colorectal cancer. This study aimed to detail the frequencies and clinicopathological features of gene fusions in colorectal cancer using a circulating tumor DNA assay.

METHODS Circulating tumor DNA samples in patients with advanced colorectal cancer were analyzed at 4,581 unique time points using a validated plasma-based multigene assay that includes assessment of fusions in *FGFR2*, *FGFR3*, *RET*, *ALK*, *NTRK1*, and *ROS1*. Associations between fusions and clinicopathological features were measured using Fisher's exact test. Relative frequencies of genomic alterations were compared between fusion-present and fusion-absent cases using an unpaired *t* test.

RESULTS Forty-four unique fusions were identified in 40 (1.1%) of the 3,808 patients with circulating tumor DNA detected: *RET* (n = 6; 36% of all fusions detected), *FGFR3* (n = 2; 27%), *ALK* (n = 10, 23%), *NTRK1* (n = 3; 7%), *ROS1* (n = 2; 5%), and *FGFR2* (n = 1; 2%). Relative to nonfusion variants detected, fusions were more likely to be subclonal (odds ratio, 8.2; 95% CI, 2.94 to 23.00; $P < .001$). Mutations associated with a previously reported anti–epidermal growth factor receptor (anti-EGFR) therapy resistance signature (subclonal *RAS* and *EGFR* mutations) were found with fusions in *FGFR3* (10 of 12 patients), *RET* (nine of 16 patients), and *ALK* (seven of 10 patients). For the 27 patients with available clinical histories, 21 (78%) had EGFR monoclonal antibody treatment before fusion detection.

CONCLUSION Diverse and potentially actionable fusions can be detected using a circulating tumor DNA assay in patients with advanced colorectal cancer. Distribution of coexisting subclonal mutations in *EGFR*, *KRAS*, and *NRAS* in a subset of the patients with fusion-present colorectal cancer suggests that these fusions may arise as a novel mechanism of resistance to anti-EGFR therapies in patients with metastatic colorectal cancer.

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ASSOCIATED CONTENT

Appendix

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INTRODUCTION

Fusions resulting in activation of proto-oncogenes lead to pathologic proliferation in a variety of malignancies and can serve as potential therapeutic targets.[1,2] Although selective kinase inhibitors have become standard-of-care therapies for *ALK*- and *ROS1*-rearranged non–small-cell lung cancers (NSCLCs), no US Food and Drug Administration–approved targeted therapies for fusions in colorectal cancer (CRC) were available until the recent approval of larotrectinib for any advanced solid tumor with *NTRK* fusions.[3] In two small series, the ALK inhibitors ceritinib and entrectinib demonstrated benefit in patients with CRC harboring *ALK* fusions.[4,5] In addition, rearranged during transfection (RET) inhibitors have shown preclinical promise in *RET* fusions both in vitro and in vivo for RET-fusion CRC.[6,7] Using tissue-based assays, fusions have been reported in approximately 1% of patients with CRC but are more common in right-sided, *RAS* wild-type, microsatellite instability–high (MSI-H) colon cancers.[8-12] However, no studies to date have comprehensively described the prevalence and genomic landscape of fusions in CRC using circulating tumor DNA (ctDNA).

When measured by ctDNA, early truncal mutations tend to occur at higher variant allele fractions (VAFs) compared with mutations acquired later in disease progression.[13] ctDNA may thereby uncover the genomic evolution of mechanisms of treatment resistance, because subclonal mutations not initially detected in primary tumor specimens may become detectable after selective pressure of targeted therapies.[14] For example, using ctDNA assays, *KRAS*, *NRAS*, *MET*, *ERBB2*, *EGFR*, *FGFR1*, and *MAP2K1* mutations have been identified as mechanisms of resistance to anti-EGFR antibody therapy in patients

CONTEXT

Key Objective

Using a circulating tumor DNA assay, we aimed to describe the frequencies and clinicopathological features of gene fusions in patients with advanced colorectal cancer.

Knowledge Generated

A circulating tumor assay was able to detect actionable fusions in patients with colorectal cancer at a prevalence of 1.1%. Genomic signatures previously associated resistance to anti–epidermal growth factor receptor therapies were found in patients with fusions.

Relevance

Coexisting subclonal mutations in patients with fusion-present colorectal cancer implicate fusions as a previously unreported, novel mechanism of resistance to anti–epidermal growth factor receptor therapies in patients with metastatic colorectal cancer.

with CRC.[15-20] Activating fusions have been found to be associated with resistance to EGFR-targeted therapies in several malignancies, including NSCLC and head and neck cancer.[21-24]

To our knowledge, no prior studies have detailed the use of a ctDNA assay in a large series for detection of oncogenic fusions in CRC. Here, we aimed to use next-generation sequencing (NGS) data from a ctDNA assay to expand the clinical use of fusion testing in a cohort of patients with advanced and typically pretreated CRC.

METHODS

Patient Population

A cohort of 4,289 consecutive patients with stage III or IV CRC underwent molecular profiling at 4,581 unique time points between February 2015 and December 2017 using a validated plasma-based 68-, 70-, or 73-gene ctDNA NGS assay (Guardant360, Guardant Health, Redwood City, CA), as previously detailed.[25,26] This assay was performed in a Clinical Laboratory Improvement Amendments–certified, College of American Pathologists–accredited, and New York State Department of Health–approved setting using a targeted digital sequencing panel with the ability to detect single-nucleotide polymorphisms, insertions/deletions (indels), amplifications, and fusions. The 68-gene panel included *ALK*, *RET*, *ROS1*, and *NTRK1* fusions, and the 70- and 73-gene panels also tested for *FGFR2* and *FGFR3* fusions (Appendix Table A1). There was no difference in the exon coverage of *KRAS*, *NRAS*, *PIK3CA*, *BRAF*, or *EGFR* among these three panels. Germline variants were filtered out as previously described.[27] The reportable range for single nucleotide variants (SNVs), indels, fusions, and amplifications is greater than 0.04% per two molecules, greater than 0.02% per one molecule, greater than 0.04% per two molecules, and greater than 2.12 copies, respectively, with a greater than 99.9999% per-position analytic specificity.[26] Clinical information was obtained from test request forms and confirmed by pathology and medical reports and from treating clinicians when available.

This research was approved by the Quorum Institutional Review Board for the generation of de-identified data sets for research. All work was conducted in accordance with the Declaration of Helsinki. Human investigations were performed after approval by a local human investigations committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services, where appropriate.

ctDNA Assay Analysis

VAF was calculated as the ratio of the number of ctDNA molecules harboring a mutation relative to the total number of molecules (variant plus wild type) for a given gene locus. To annotate a given alteration by clonality, relative VAF (rVAF) was assessed by normalizing the VAF to the maximum VAF of all aberrations detected within a given plasma sample, adjusting for copy number amplification as previously described.[13] For the purpose of this study, clonal aberrations were defined as rVAF of 0.5 to 1, subclonal aberrations as rVAF between 0.1 and 0.5, and subclonal minor as rVAF less than 0.1.[13]

Associations between the presence of fusions and clinicopathological features were evaluated using a Fisher's exact test (SPSS, version 24.0; La Jolla, CA). Relative frequencies of genomic alterations (point mutations, indels, and splice variants) were compared between fusion-present and fusion-absent cases using an unpaired *t* test.

RESULTS

Occurrence of Fusions in a ctDNA Assay

The median age at time of ctDNA testing was 59 years (interquartile range, 50-69 years). A total of 1,909 patients (44.5%) were female. Of the 3,808 patients with detectable alterations at any time point (Fig 1A), 44 unique fusions were reported in 40 patients (1.1% prevalence). These fusions detected *RET* (n = 16; 36% of all fusions detected), *FGFR3* (n = 12; 27%), *ALK* (n = 10; 23%), *NTRK1* (n = 3; 7%), *ROS1* (n = 2; 5%), and *FGFR2* (n = 1; 2%). When examining the prevalence of fusions by rearrangement

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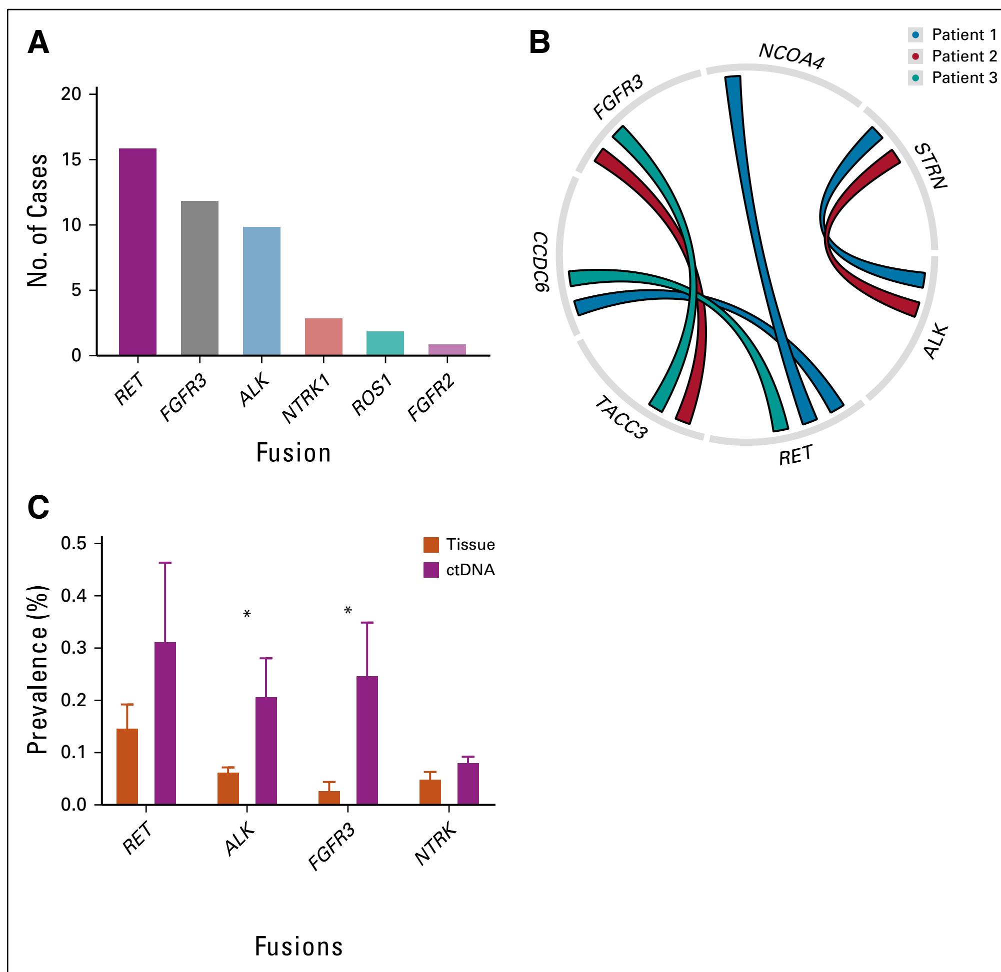

FIG 1. Prevalence of fusions with circulating tumor DNA (ctDNA). (A) Overall prevalence of fusions. (B) Specimens with co-occurring fusions. (C) Fusion prevalence in ctDNA-based assay compared with tissue-based assay with 95% upper CIs. (*) Indicates fusions with statistically significant differences in prevalence between tissue and ctDNA.

partner, the most commonly detected fusions were the *FGFR3-TACC3* (n = 12) and *NCOA4-RET* (n = 9) fusions (Appendix Table A2). Co-occurring fusions were found in three of 40 patients (Fig 1B).

The prevalences of *ALK* and *FGFR3* fusions were significantly higher in this ctDNA cohort compared with a previously reported cohort of 4,422 CRC tissue specimens undergoing comprehensive NGS genomic profiling ($P = .04$, $P = .01$, respectively).There was no difference in frequencies of *RET* or *NTRK* fusions between ctDNA and tissue assays (Fig 1C; Table 1).[28]

TABLE 1. Fusion Prevalence in ctDNA-Based Assay Compared With Tissue-Based Assay

Assay	RET	ALK	FGFR3	NTRK
Tissue: No. of fusions present	8	3	1	2
Tissue: No. of samples tested	4,422	4,422	4,422	4,422
ctDNA: No. of fusions present	16	10	12	3
ctDNA: No. of samples tested	3,808	3,808	3,808	3,808

Abbreviation: ctDNA, circulating tumor DNA.

Genomic Profiling of Fusion-Positive Patients

Clinicopathology history was available for a subset of patients (Table 2; Appendix Table A3). Because this was a retrospective review of clinically treated patients, tissue testing methodology varied over time and across different practices. At least some of the molecular data from tissue testing collected at the time of initial diagnosis was available for 24 of 40 ctDNA fusion-positive patients, eight of whom had comprehensive NGS in which the presence of fusions was assessed. The median time between tissue testing and ctDNA collection was 24.1 months (range, 0.67 to 92 months; n = 22). From the available clinical and tissue data, nine of 27 (33%) were right-sided, tumors were predominantly *KRAS* wild type (n = 23 of 24; 96%), with no concurrent *NRAS* or $BRAF^{V600E}$ mutations, and three of 22 (14%) were MSI-H (Table 2; Table A3). Interestingly, in 11 of the 23 patients with tissue *RAS* wild-type status, a *RAS* mutation was detected in ctDNA. Similarly, in two of the

TABLE 2. Tissue Molecular and MSI Testing at Time of Metastatic CRC Diagnosis

Molecular Mutation and MSI Status	Positive (or MSI-H)	WT (or MSS)	Not Tested	Unknown	Sum	Total Known	Alteration/All Tested Cases
KRAS							
Overall	1	23	0	16	40	24	0.0416
Expanded (codons 12, 13, 59, 61, 117, 146; eg, exons 2-4)		9					
Limited (known codons)		6					
Codon 12		6					
Codon 13		6					
Codon 61		5					
Limited NOS		3					
Unknown		5					
NRAS							
Overall	0	17	5	18	40	17	0
Expanded (codons 12, 13, 59, 61, 117, 146; eg, exons 2-4)		9					
Limited (known codons)		1					
Codon 12		0					
Codon 13		0					
Codon 61		1					
Limited NOS		1					
Unknown		6					
BRAF V600E							
Overall	0	16	6	18	40	16	0
Fusion							
Overall	2	5	11	21	40	7	0.286
MSI							
Overall	3	19	2	16	40	22	0.1363
Loss of MLH1/PMS2	2						
Loss of PMS2	1						

Abbreviations: MSI, microsatellite instability; MSI-H, MSI-high; MSS, microsatellite stable; NOS, not otherwise specified; WT, wild type.

16 patients with *BRAF*^{V600E} tissue wild-type status, *BRAF*^{V600E} was detected in ctDNA. Among the eight patients with tissue NGS available, only two had the matched fusion detected. Cumulatively, the data suggest that a sizable proportion of the ctDNA fusion-positive population may have had *RAS/RAF* mutations and/or the fusion present at levels below the limit of detection in tissue or in a subclone of the tumor tissue that was not sampled for testing.

The frequency of amplifications, indels, and SNVs in clinically relevant cancer genes detectable using the blood-based NGS assay were compared between fusion-positive and fusion-negative samples (Fig 2A). There was no association between the presence of a fusion and coexisting mutation in *KRAS*, *NRAS*, or *BRAF*. Furthermore, co-occurring mutations were more likely in *EGFR* (odds ratio [OR], 3.66; 95% CI, 1.97 to 6.84; $P < .001$), *MET* (OR, 2.56; 95% CI, 1.30 to 5.04; $P < .01$), and *FGFR1* (OR, 2.46; 95% CI, 1.20 to 5.06; $P = .01$) for specimens with fusions, when compared with nonfusion cases (Fig 2A).

Prior treatment histories were available for only 27 patients, the majority (n = 21; 78%) of whom did have prior exposure to one or more EGFR monoclonal antibodies as treatment of metastatic CRC at the time of ctDNA collection (Appendix Table A4; Appendix Fig A1). Therefore, we next explored if fusions were associated with a previously validated genomic signature associated with CRC progression on prior anti-EGFR therapies, because treatment histories were not available for the entire fusion cohort.[16,29]

Anti-EGFR Signature

ctDNA genomic features of progression on prior cetuximab or panitumumab include the presence of subclonal *RAS* mutation (VAF < 50% of the maximum VAF in the sample), multiple concurrent *RAS* mutations, and/or *EGFR* mutations.[29]

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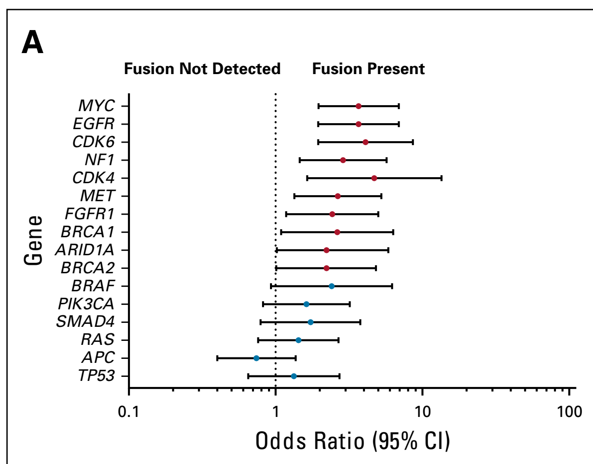

FIG 2. Co-occurring mutations in fusion patients. (A) Gene mutations associated with fusion presence. (B) *EGFR*, *RAS*, and *BRAF* amplifications, indels, and mutations occurring in fusion patients in circulating tumor DNA (ctDNA) and matched tissue samples. Blank cells indicate no mutation detected (for circulating free DNA [cfDNA] results) or not available (for tissue results and prior anti-epidermal growth factor receptor [EGFR] monoclonal antibody [mAb]). (*) Clinical history verified by ordering health care provider. (†) Patients had multiple samples drawn; figure provides a summary of unique alterations (alts) detected across all fusion-positive samples. +, KRAS G13D positive; ECD, extracellular domain; H, MSI-high; L, left; mAb, monoclonal antibody; MSI, microsatellite instability; N, no; R, right; Tr, transverse; S, MSI-stable; WT, wild type; Y, yes.

In a previously validated large cohort of patients with metastatic CRC with and without anti-EGFR exposure, the presence of any one of these variables was highly predictive of prior anti-EGFR exposure (positive predictive value, 98.3%; specificity, 98.7%).[29]

In this series, 24 of 40 (60%) fusion-positive patients had subclonal *RAS* mutation (rVAF of $<$ 50%), any *EGFR* mutation, or multiple concurrent *RAS* mutations. Fifteen of 40 (38%) had two or more of these. Mutations associated with this anti-EGFR therapy resistance signature were found with fusions in *FGFR3* (10 of 12 patients), *RET* (nine of 16 patients), and *ALK* (seven of 10 patients), including two of the patients with multiple fusions (Fig 2B). Among the 24 fusion-positive patients with mutations associated with this anti-EGFR therapy resistance signature, 19 had known treatment histories (Fig A1). Of these 19 patients, 16 (84%) patients were confirmed to have prior exposure to anti-EGFR therapy. The median duration of exposure to treatment with an anti-EGFR agent was 8.5 months (range, 2 to 17 months; Appendix Tables A4 and A5).

The presence of an anti-EGFR signature was associated with fusions occurring at lower rVAF (median, 0.01 *v* 0.19; P = .036; Fig 3). Furthermore, the low rVAFs of co-occurring *RAS*, *EGFR*, and *BRAF*^{V600E} mutations were consistent with subclonal genomic events occurring later in tumorigenesis (Appendix Fig A2). Among the six patients with an anti-EGFR resistance signature who had comprehensive genomic profiling results available from tissue, four were wild type at the time of initial diagnosis of CRC for the corresponding fusion and/or *RAS/RAF* alterations that were later detected in ctDNA, consistent with these genomic events being acquired later in tumorigenesis.

DISCUSSION

To our knowledge, this is the largest case series describing fusion-positive cases in CRC (whether in tissue or plasma) and demonstrates that fusions in patients with CRC can be identified using a ctDNA assay. Here, fusions were detected at a prevalence of approximately 1% in patients with advanced CRC, similar to fusion prevalence using orthogonal tissue-based assays in separate series of patients with CRC.[8,9] All fusions identified are potentially actionable with available targeted drugs. Thus, this ctDNA approach has the potential to allow clinicians to consider additional studies with novel therapeutic combinations for patients with metastatic CRC in future trial settings.

Our data provide new evidence that fusions, particularly involving *FGFR3* or *RET*, may contribute to anti-EGFR therapy resistance in CRC. Here, the majority of the fusions were subclonal. On the basis of previously validated genomic signatures in this setting, we hypothesize that fusions may arise as a novel, unreported mechanism with anti-EGFR therapy resistance, given the clinicopathologic data and frequent co-occurrence with subclonal *RAS* and *EGFR* mutations in ctDNA. The profile of concomitant *EGFR* mutations and subclonal *RAS* mutations mirrors prior studies that have shown associations between these mutations and post-EGFR resistance.[29] Interestingly, prior series performed in tissue have associated fusions with *RAS* wild-type CRC tumors.[9-12] In our series, 23 of 24 (96%) of the ctDNA fusion–positive patients with tissue testing available for *RAS* mutational status were *RAS* wild type, whereas 25 of 44 (57%) of fusion-positive ctDNA samples in our series had one or more *RAS* mutations. We reconcile these findings on the basis of the greater sensitivity to detect low allele frequency often not detectable with tissue-based assays. Furthermore, tissue specimens are often obtained at surgical resections, before a multiple number of sequential lines of systemic therapy, and therefore before exposure to selective pressures that mediate acquisition of resistance mechanisms. The majority of blood samples obtained in this cohort of patients with CRC came from treatment-refractory individuals seeking clinical trial options who frequently had been exposed to anti-EGFR therapies. Thus, the occurrence of subclonal resistance alterations in ctDNA accounted for differences in the tumor genomic profiles of advanced, typically heavily pretreated cancers, relative to the less-mutated genomic profiles of the tumor taken before therapy initiation. In this series, only one patient had a fusion detected in pretreatment tumor tissue and subsequently had anti-EGFR therapy but did not have a clinical response.

B

Patient	Fusion	*EGFR* ECD													*KRAS*				*NRAS*				Amplifications			Total No. cfDNA Alts	Anti-EGFR Signature	Tissue Results					Prior EGFR mAb
		A86V	F404I	Q432P	V441D/G	S442R	S464L	G465E/R/V	I491K	S492R	V536M	G640E	*BRAF* V600E	G12A/C/D/V	G13D	Q61H/L	K117N	G12C	Q61H/K/R	*MAP2K1*	*MAP2K2*	*MAPK3*	*EGFR*	*BRAF*	*KRAS*			*RAS*	*BRAF* V600E	Fusion	Sidedness	MSI	
1	*RETx2 + ALK*																									22	Y	-	-	-	-	-	Y
2*	*RET + FGFR3*																									7	-	WT	WT	WT	L	S	Y
3*	*ALK + FGFR3*																									7	Y	WT	WT	-	L	S	Y
4*	*RET*																									37	Y	WT	WT	+	Tr	H	Y
5*	*RET*																									21	Y	WT	WT	+	Tr	H	N
6	*RET*																									20	Y	-	-	-	-	-	Y
7*	*RET*																									20	Y	WT	WT	WT	R	S	Y
8†*	*RET*																									19	Y	WT	WT	WT	R	H	N
9*	*RET*																									14	-	G13D	-	-	Tr	-	N
10	*RET*																									12	Y	-	-	-	-	-	-
11	*RET*																									8	Y	-	-	-	-	-	-
12	*RET*																									6	-	WT	WT	-	R	S	N
13*	*RET*																									6	Y	WT	-	WT	L	S	Y
14	*RET*																									4	-	-	-	-	R	-	-
15	*RET*																									2	-	WT	WT	-	Tr	S	-
16*	*RET*																									2	-	WT	-	-	R	S	Y
17*	*FGFR3*																									30	Y	WT	WT	WT	L	S	Y
18*	*FGFR3*																									24	Y	WT	WT	-	-	S	Y
19	*FGFR3*																									18	Y	-	-	-	-	-	Y
20*	*FGFR3*																									14	Y	WT	WT	-	L	S	Y
21*	*FGFR3*																									12	Y	WT	-	-	L	S	Y
22*	*FGFR3*																									12	Y	WT	-	-	L	S	Y
23	*FGFR3*																									12	Y	-	-	-	-	-	-
24*	*FGFR3*																									11	Y	WT	WT	-	L	S	N
25*	*FGFR3*																									8	Y	WT	WT	-	-	S	Y
26	*FGFR3*																									6	-	-	-	-	L	-	-
27	*FGFR2*																									19	-	-	-	-	-	-	-
28	*ALK*																									32	Y	WT	-	-	L	S	Y
29	*ALK*																									29	Y	-	-	-	-	-	-
30*	*ALK*																									15	Y	WT	-	-	L	S	Y
31	*ALK*																									12	Y	-	-	-	-	-	-
32*	*ALK*																									12	-	WT	WT	WT	R	S	Y
33*	*ALK*																									7	Y	WT	WT	-	L	S	Y
34	*ALK*																									5	-	WT	WT	-	R,L	S	Y
35†	*ALK*																									3	-	-	-	-	R	-	-
36*†	*NTRK1*																									10	-	WT	-	-	L	-	N
37	*NTRK1*																									5	-	-	-	-	R	-	-
38	*NTRK1*																									3	-	-	-	-	-	-	Y
39	*ROS1*																									8	-	-	-	-	-	-	-
40	*ROS1*																									3	-	-	-	-	L	-	-

FIG 2. (Continued).

Additional investigation into whether fusions also cause primary resistance to anti-EGFR therapy is warranted.

To lend additional support to this association between fusions and resistance to anti-EGFR antibodies, we confirmed the clinical histories of patients with fusion-detected CRC. In those patients with prior treatment data available, 21 of 27 (78%) had previous exposure and progression on anti-EGFR antibodies. Thus, these data further support the notion that subclonal fusions, here identified by ctDNA, may arise after treatment with anti-EGFR antibodies and may represent a novel mechanism of resistance in CRC to these agents.

Our findings in CRC are consistent with previously reported series linking activating fusions as mechanisms of acquired resistance to targeted therapies in other malignancies.[21-24] For example, *RET* fusions were found in patients with NSCLC after the EGFR tyrosine kinase inhibitor (TKI) osimertinib.[23] Previous studies have shown that *FGFR3*

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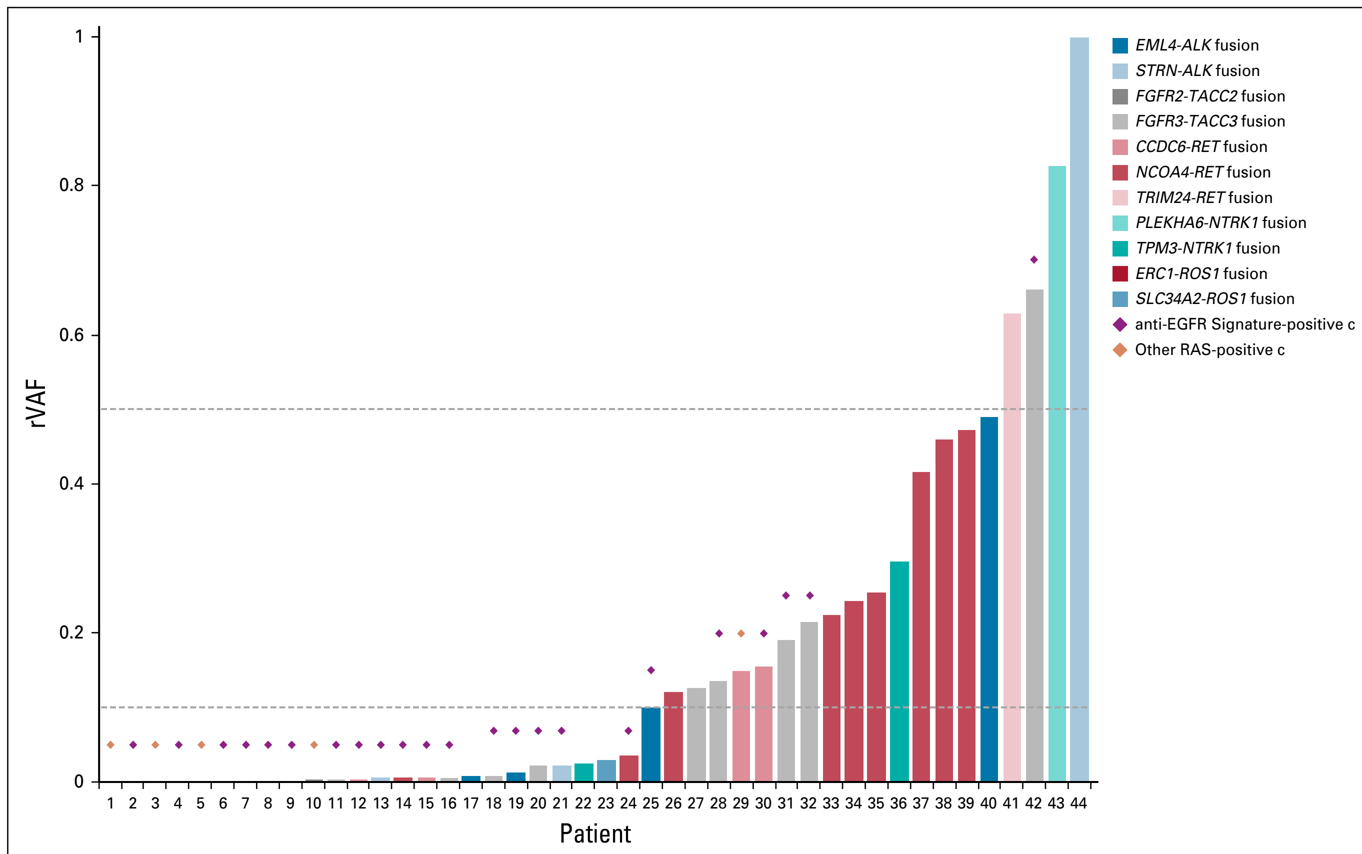

FIG 3. Distribution of copy-number adjusted relative variant allele fraction (rVAF) for fusion-present samples. Values above horizontal line at rVAF 0.5 indicate clonal fusions, values between 0.1 and 0.5 indicate subclonal fusions, and values below horizontal line at 0.1 indicate subclonal minor fusions.

fusions may substitute for EGFR signaling, which provides a hypothesized rationale for a mechanism of acquired resistance to anti-EGFR therapy.[21] All of the fusions detected in this series are predicted to lead to the generation of a chimeric protein involving fusion of a tyrosine kinase domain with a partner protein that enhances its activation, thereby promoting downstream signaling of the mitogen-activated protein kinase (MAPK) pathway.[30,31] Activation of this alternative MAPK signaling pathway bypasses the reduction in MAPK signaling afforded by anti-EGFR antibodies, thus providing plausible biologic rationale for the association of fusion anti-EGFR therapy resistance.[32]

Furthermore, alterations in *MET* and *FGFR1* were also observed more commonly in patients with fusions. Such alterations have been previously reported as acquired mechanisms of resistance to anti-EGFR therapies in CRC.[20,33] Collectively these and our data point to a diverse, heterogeneous landscape of potential resistance mechanisms adapted by *RAS* wild-type CRC tumors to overcome EGFR blockade.

Fusions represent a potentially actionable therapeutic target in the anti-EGFR resistance setting. Dual pathway suppression with the RET inhibitor BLU-667 and an EGFR TKI demonstrated antitumor activity both in cell lines and clinically in patients with EGFR-mutant NSCLC who had *RET* fusions after disease progression while receiving TKIs.[23] Importantly, fusions were often seen co-occurring with multiple other known acquired mechanisms of resistance to anti-EGFR therapy in this series, which points to a diverse, heterogeneous landscape of potential resistance mechanisms adapted by *RAS* wild-type CRC tumors to overcome EGFR blockade. Therefore, although targeting subclonal fusions alone may be only partially successful, multipathway suppression may be a promising avenue of additional investigation, possibly in combination with anti-EGFR therapies. Such strategies would need to be highly individualized, given the diversity of resistance mechanisms, and could be informed by comprehensive ctDNA testing, especially because serial tissue biopsies are less feasible in patients with advanced cancer.

In several previous data sets using tissue-based assays, fusions in patients with CRC were associated with MSI-H cancers.[9-12] Although rates of MSI-H and right-sided tumors in our data set were similar to average rates reported in advanced CRCs, a proportion of the fusion-positive patients in this series are suspected to have acquired the fusion after selective pressure from anti-EGFR therapy, and therefore the fusion may have been present in the primary tumor at levels too low to be associated with MSI-H status. In both cases in

this series where the fusion was tested for and detected in tissue, the tumors were found to be MSI-H.

One of the limitations of this data analysis is that complete clinicopathologic features were not available for all patients, given the retrospective nature of the study, and therefore we were unable to obtain clinical histories from all patients with fusions. However, using a previously validated method,[29] the majority of fusion-positive patients had at least one variable, which was highly predictive of prior anti-EGFR exposure. In addition, among the patients with known treatment history and this signature, the majority were indeed confirmed to have prior anti-EGFR therapy, thus internally validating the efficacy of this genomics-first strategy to identify likely resistance cases.

We also did not have matched pre- and post-treatment tissue and plasma for orthogonal and serial profiling to confirm which fusions and other co-occurring mutations were acquired/selected for after anti-EGFR therapy versus those present as truncal/clonal events. For the majority of our patients, we do not have access to the tissue or pretreatment plasma for additional NGS analysis based on the retrospective nature of study. However, genomic events that are acquired during cancer progression tend to have lower relative VAF in ctDNA than do early truncal mutations, such as those in tumor suppressor genes or clonal RAS mutations.[13] In our series, fusions occurring at low rVAF tended to be found in samples containing other genomic mechanisms of anti-EGFR therapy resistance, which is consistent with our hypothesis that some fusions in CRC occur at subclonal levels that are undetectable in pretreatment tissue but are selected for and become detectable in ctDNA after anti-EGFR therapy resistance. The quantitative nature of ctDNA can therefore not only characterize the fusion identity but also provide insight into the clonal contribution via a single blood draw. Another limitation is that the VAF may be affected by biologic factors, such as the degree of tumor shedding, as well as technical factors, including that fusions are more difficult to detect by NGS and in ctDNA samples than SNVs. Taken together, the fusion prevalences and VAFs observed in this study may be lower than actual because of these technical reasons.

In conclusion, actionable fusions were able to be detected at low frequencies but at similar frequencies to the historical tissue-based NGS approach in a large series of patients with CRC using a ctDNA assay. The distribution of coexisting subclonal mutations in *EGFR*, *KRAS*, and *NRAS* in fusion-present CRC cases matches genomic profiles of CRC tumors after progression on prior anti-EGFR therapy in tumors initially identified as *RAS* wild type using a less-sensitive tissue-based assay. Actionable fusions may therefore represent a newly reported mechanism of acquired resistance after anti-EGFR therapies. Testing ctDNA in patients to detect fusions as targetable drivers and/or resistance biomarkers is warranted and may carry important implications for the treating oncologist to identify novel therapeutic approaches.

AFFILIATIONS

[1]The University of Texas MD Anderson Cancer Center, Houston, TX
[2]GuardantHealth, Redwood City, CA
[3]BC Cancer, Vancouver, British Columbia, Canada
[4]Tennessee Oncology Sarah Cannon Research Institute, Nashville, TN
[5]Northwestern University Feinberg School of Medicine, Chicago, IL
[6]Cone Health Cancer Center, Greensboro, NC
[7]University of San Diego Moores Cancer Center, La Jolla, CA
[8]The University of Tennessee West Cancer Center, Memphis, TN
[9]Cedars-Sinai Medical Center, Los Angeles, CA
[10]University of Pennsylvania, Philadelphia, PA
[11]The University of Arizona Cancer Center, Tucson, AZ
[12]Pacific Medical Center of Hope, Fresno, CA
[13]Washington University School of Medicine, St Louis, MO
[14]University of California Los Angeles, Los Angeles, CA

CORRESPONDING AUTHOR

Van Morris, MD, Department of Gastrointestinal Medical Oncology, University of Texas MD Anderson Cancer Center, 1400 Holcombe Blvd, Unit 426, Houston, TX 77030; Twitter: @VanMorrisMD; e-mail: vkmorris@mdanderson.org.

EQUAL CONTRIBUTION

K.C. and T.A.R. contributed equally to this work.

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AUTHOR CONTRIBUTIONS

Conception and design: Katherine Clifton, Thereasa A. Rich, Victoria M. Raymond, Allan Andresson Lima Pereira, Jonathan M. Loree, Axel Grothey, Mohammad Amin Nezami, Richard Lanman, Scott Kopetz, Van Morris

Financial support: Van Morris

Administrative support: Victoria M. Raymond, Mohammad Amin Nezami, Scott Kopetz

Provision of study material or patients: Victoria M. Raymond, Todd M. Bauer, Young Kwang Chae, Paul Fanta, Axel Grothey, Andrew Hendifar, Daruka Mahadevan, Benjamin Tan, Zev A. Wainberg

Collection and assembly of data: Thereasa A. Rich, Victoria M. Raymond, Todd M. Bauer, Young Kwang Chae, Gary Sherrill, Paul Fanta, Andrew Hendifar, Daruka Mahadevan, Benjamin Tan, Zev A. Wainberg, Richard Lanman, Van Morris

Data analysis and interpretation: Thereasa A. Rich, Christine Parseghian, Victoria M. Raymond, Arvind Dasari, Jason Willis, Jonathan M. Loree, Todd M. Bauer, Young Kwang Chae, Paul Fanta, Andrew Hendifar, David Henry, Daruka Mahadevan, Zev A. Wainberg, Richard Lanman

Manuscript writing: All authors

Final approval of manuscript: All authors

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Katherine Clifton
Employment: WellCare (I)
Stock and Other Ownership Interests: WellCare (I)

Thereasa A. Rich
Employment: Guardant Health
Stock and Other Ownership Interests: Guardant Health, Myriad Genetics

Victoria M. Raymond
Employment: TrovaGene, Guardant Health
Stock and Other Ownership Interests: TrovaGene, Guardant Health

Arvind Dasari
Consulting or Advisory Role: Ipsen, AbbVie/Stemcentrx, Novartis, Voluntis, Lexicon
Research Funding: Novartis, eFFECTOR Therapeutics, Eisai, Hutchison MediPharma, Merck, Guardant Health

Jonathan M. Loree
Consulting or Advisory Role: Taiho Pharma Canada, Ipsen, Novartis, Bayer, Amgen

Todd M. Bauer
Employment: Tennessee Oncology
Consulting or Advisory Role: Ignyta (Inst), Guardant Health, Loxo, Pfizer, Moderna Therapeutics (Inst), Pfizer (Inst), Exelixis
Speakers' Bureau: Bayer
Research Funding: Daiichi Sankyo (Inst), MedPacto (Inst), Incyte (Inst), Mirati Therapeutics (Inst), MedImmune (Inst), AbbVie (Inst), AstraZeneca (Inst), Leap Therapeutics (Inst), MabVax Therapeutics (Inst), Stemline Therapeutics (Inst), Merck (Inst), Eli Lilly (Inst), GlaxoSmithKline (Inst), Novartis (Inst), Pfizer (Inst), Genentech (Inst), Deciphera Pharmaceuticals (Inst), Merrimack (Inst), Immunogen (Inst), Millennium (Inst), Ignyta (Inst), Calithera Biosciences (Inst), Kolltan Pharmaceutical (Inst), Principia Biopharma (Inst), Peleton (Inst), Immunocore (Inst), Roche (Inst), Aileron Therapeutics (Inst), Bristol-Myers Squibb (Inst), Amgen (Inst), Moderna Therapeutics (Inst), Sanofi (Inst), Boehringer Ingelheim (Inst), Astellas Pharma (Inst), Five Prime Therapeutics (Inst), Jacobio Pharmaceuticals (Inst), Top Alliance BioSciences (Inst), Loxo (Inst), Janssen (Inst), Clovis Oncology (Inst), Takeda (Inst), Karyopharm Therapeutics (Inst), Onyx (Inst), Phosplatin Therapeutics (Inst), Foundation Medicine (Inst), ARMO BioSciences (Inst)
Travel, Accommodations, Expenses: Astellas Pharma, AstraZeneca, Celgene, Clovis Oncology, EMD Serono, Genentech, Eli Lilly, Merck, Novartis, Pharmacyclics, Sysmex

Young Kwang Chae
Consulting or Advisory Role: Foundation Medicine, Boehringer Ingelheim, Biodesix, Counsyl, AstraZeneca, Guardant Health, Takeda, Genentech, ImmuneOncia, Hanmi
Speakers' Bureau: Genentech, Merck, AstraZeneca, Eli Lilly
Research Funding: AbbVie, Bristol-Myers Squibb, Lexent Bio, Freenome, Biodesix
Travel, Accommodations, Expenses: Hanmi

Axel Grothey
Honoraria: Elsevier, Aptitude Health, Imedex
Consulting or Advisory Role: Genentech (Inst), Bayer (Inst), Bristol-Myers Squibb (Inst), Eli Lilly (Inst), Boston Biomedical (Inst), Amgen (Inst), Array BioPharma (Inst), Guardant Health (Inst), Daiichi Sankyo (Inst)
Research Funding, Genentech (Inst), Bayer (Inst), Pfizer (Inst), Eisai (Inst), Eli Lilly (Inst), Boston Biomedical (Inst), Daiichi Sankyo (Inst), Array BioPharma (Inst)
Travel, Accommodations, Expenses: Genentech, Bayer, Bristol-Myers Squibb, Boston Biomedical, Amgen, Boehringer Ingelheim, Merck Sharp & Dohme

Andrew Hendifar
Consulting or Advisory Role: Novartis, Ipsen, Perthera, Celgene, AbbVie
Research Funding: Ipsen
Travel, Accommodations, Expenses: Halozyme

David Henry
Honoraria: Amgen, AMAG Pharmaceuticals
Consulting or Advisory Role: AMAG Pharmaceuticals, Amgen
Research Funding: Amgen
Travel, Accommodations, Expenses: Amgen, AMAG Pharmaceuticals

Daruka Mahadevan
Honoraria: Guardant Health, Caris Life Sciences
Speakers' Bureau: Guardant Health, Caris Life Sciences
Travel, Accommodations, Expenses: Guardant Health, Caris Life Sciences

Mohammad Nezami
Employment: Orange Coast Medical Center of Hope
Stock and Other Ownership Interests: Sahel Oncology

Benjamin Tan
Consulting or Advisory Role: Genentech
Research Funding: Genentech, Eli Lilly, Pfizer, Bayer, Eisai, Exelixis, Merck Serono, Tyrogenex, SillaJen, Boehringer Ingelheim

Zev A. Wainberg
Consulting or Advisory Role: Array BioPharma, Five Prime Therapeutics, Novartis, Eli Lilly, Merck, Merck KGaA, Bristol-Myers Squibb, Genentech, Bayer
Research Funding: Novartis (Inst), Plexxikon (Inst), Pfizer (Inst), Merck (Inst), Five Prime Therapeutics (Inst)
Travel, Accommodations, Expenses: Genentech

Richard Lanman
Employment: Guardant Health, Veracyte
Leadership: Guardant Health, Biolase
Stock and Other Ownership Interests: Guardant Health, Biolase, Forward Medical
Research Funding: Guardant Health

Scott Kopetz
Stock and Other Ownership Interests: MolecularMatch, Navire
Consulting or Advisory Role: Roche, Genentech, EMD Serono, Merck, Karyopharm Therapeutics, Amal Therapeutics, Navire Pharma, Symphogen, Holy Stone, Biocartis, Amgen, Novartis, Eli Lilly, Boehringer Ingelheim, Boston Biomedical, AstraZeneca/MedImmune, Bayer Health, Pierre Fabre, EMD Serono
Research Funding: Amgen (Inst), Sanofi (Inst), Biocartis (Inst), Guardant Health (Inst), Array BioPharma (Inst), Genentech (Inst), EMD Serono (Inst), MedImmune (Inst), Novartis (Inst), Eli Lilly (Inst)

Van Morris
Consulting or Advisory Role: Clinical Genomics, Array Biopharma
Research Funding: Bristol-Myers Squibb (Inst), Array BioPharma (Inst), EMD Serono (Inst)
Travel, Accommodations, Expenses: Array BioPharma

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APPENDIX

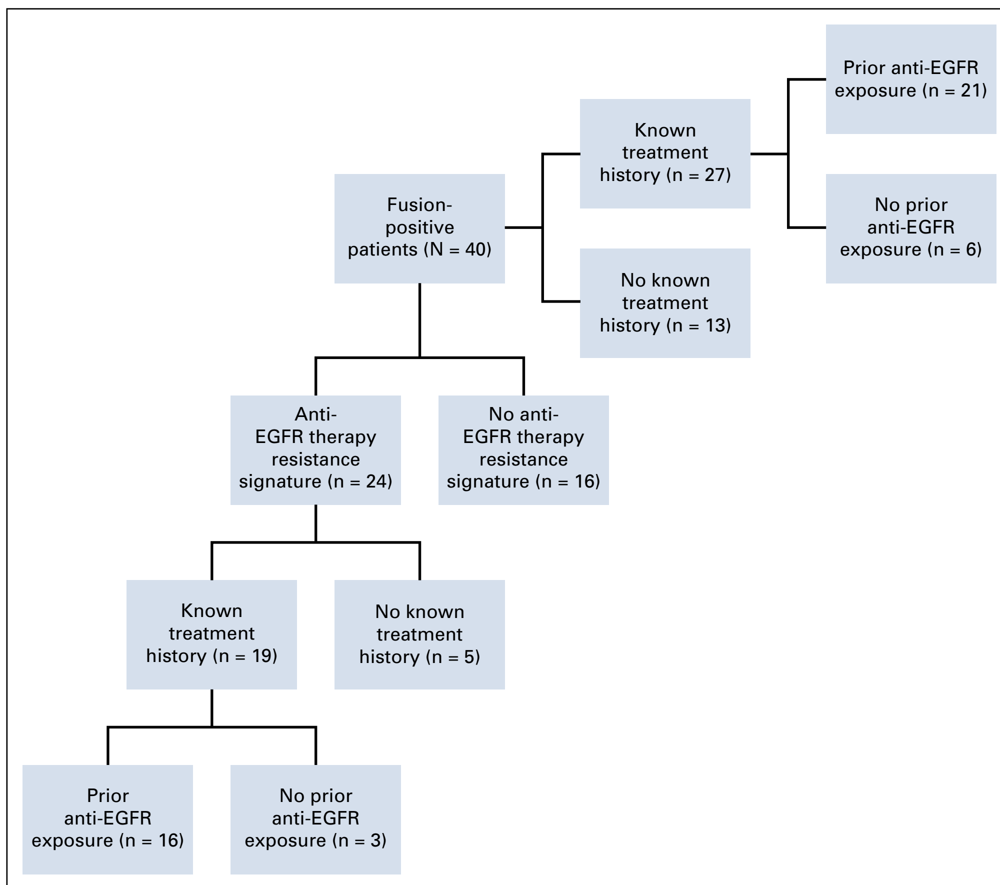

FIG A1. Consort diagram detailing fusion history and associated anti–epidermal growth factor receptor (anti-EGFR) therapy resistance signature.

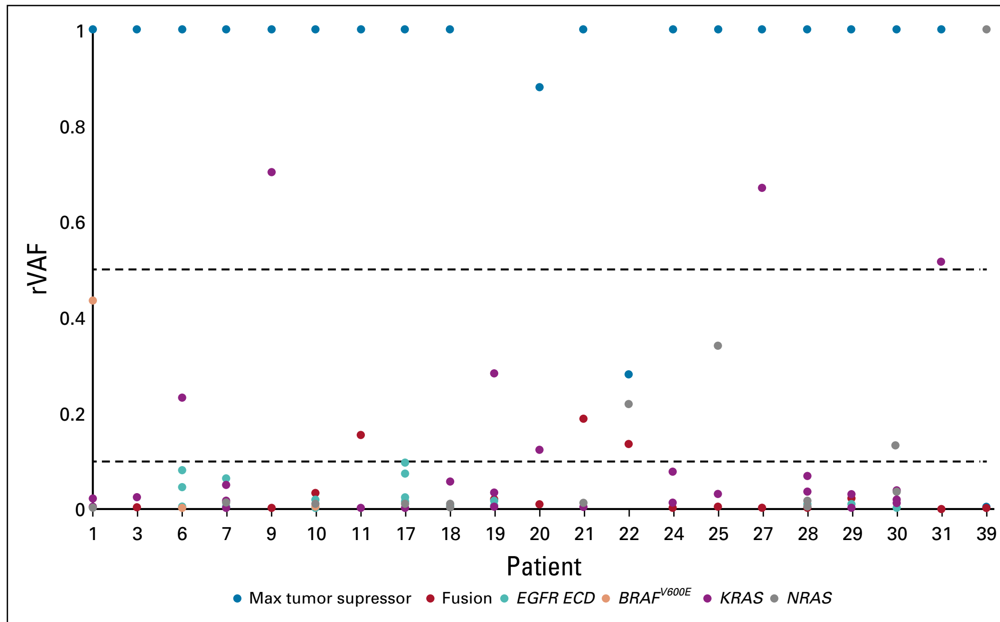

FIG A2. Relative variant allele fraction (rVAF) for 21 fusion-positive samples with *RAS* mutations and co-occurring *EGFR* extracellular domain (ECD) and *BRAF*^{V600E} mutations.

TABLE A1. Fusions Tested Using Plasma-Based Circulating Tumor DNA Next-Generation Sequencing Assay

68-Gene Panel (n = 727)	70-Gene Panel (n = 1,562)	73-Gene Panel (n = 2,293)
EML4-ALK	*EML4-ALK*	*EML4-ALK*
STRN-ALK	*STRN-ALK*	*STRN-ALK*
CCDC6-RET	*CCDC6-RET*	*CCDC6-RET*
NCOA4-RET	*NCOA4-RET*	*NCOA4-RET*
TRIM24-RET	*TRIM24-RET*	*TRIM24-RET*
ERC1-ROS1	*ERC1-ROS1*	*ERC1-ROS1*
SLC34A2-ROS1	*SLC34A2-ROS1*	*SLC34A2-ROS1*
PLEKHA6-NTRK1	*PLEKHA6-NTRK1*	*PLEKHA6-NTRK1*
TPM3-NTRK1	*TPM3-NTRK1*	*TPM3-NTRK1*
	FGFR2-TACC2	*FGFR2-TACC2*
	FGFR3-TACC3	*FGFR3-TACC3*

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TABLE A2. Prevalence by Rearrangement Partner

Fusion Partner	Patients Tested	Patients With Fusion	Prevalence of Fusion (%)
ALK			
EML4-ALK	4,289	5	0.12
STRN-ALK		5	0.12
FGFR2-TACC2	3,679	1	0.03
FGFR3-TACC3	3,679	12	0.33
NTRK1			
PLEKHA6-NTRK1	4,289	1	0.02
TPM3-NTRK1		2	0.05
RET			
CCDC6-RET	4,289	6	0.14
NCOA4-RET		9	0.20
TRIM24-RET		1	0.02
ROS1			
ERC1-ROS1	4,289	1	0.02
SLC34A2-ROS1		1	0.02

TABLE A3. Primary Tumor Location in Fusion-Positive Patients

Tumor Location	No. Patients	% (of known)
Right	8	0.30
Left	14	0.52
Both right and left	1	0.04
Transverse	4	0.15
Unknown	13	

TABLE A4. Available Clinical History of Fusion-Positive Patients

Prior Anti-EGFR Treatment	No. of Patients	Alterations/All Tested Cases
Yes	21	0.78
No	6	0.22
Unknown	13	
Duration of therapy, months (n = 12 known)		
Median	8.5	—
Minimum	2	—
Maximum	17	—
Disease progression while receiving anti-EGFR treatment at time of G360		
Yes	10	0.40
No	15	0.60
Unknown	13	—
Not applicable; was never receiving anti-EGFR	6	—
Time between last EGFR treatment and G360, months (n = 17 known)		
>1 month	8	
Median	7	
Minimum	0.7	
Maximum	23.5	

Abbreviation: EGFR, epidermal growth factor receptor; G360, Guardant360.

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TABLE A5. Anti-EGFR Treatment Characteristics

Oncoprint Number	Fusion in Pretreatment Tissue	EGFR Resistance Signature	Clinical Benefit While Receiving Anti-EGFR	Duration of Treatment (months)	Time From Treatment Stop to ctDNA (months)
2	Negative	No	Yes	7	0
3	Not performed	Yes	Yes	10	3
4	Positive	Yes	No	5	3.6
7	Negative	Yes	Yes	16	0
13	Negative	Yes	Yes	14	23.5
16	Not performed	No	No	2	3
17	Negative	Yes	Yes	10	0
20	Not performed	Yes	Yes	6	9
21	Not performed	Yes	Yes	11.5	0.7
22	Not performed	Yes	No	6	8
28	Not performed	Yes	Yes	14	13
30	Not performed	Yes	Yes	17	7
32	Negative	No	Unknown	6	0
33	Not performed	Yes	No	5	0

Abbreviations: ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor.