Clinical Cancer Research

Analysis of Cell-Free DNA from 32,989 Advanced Cancers Reveals Novel Co-occurring Activating *RET* Alterations and Oncogenic Signaling Pathway Aberrations

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Abstract

Purpose: *RET* is an emerging oncogenic target showing promise in phase I/II clinical trials. An understudied aspect of *RET*-driven cancers is the extent to which co-occurring genomic alterations exist and how they may impact prognosis or therapeutic response.

Experimental Design: Somatic activating *RET* alterations were identified among 32,989 consecutive patients with metastatic solid tumors tested with a clinical cell-free circulating tumor DNA (cfDNA) assay. This comprehensive next-generation sequencing (NGS) assay evaluates single-nucleotide variants, and select indels, fusions, and copy number gains in 68–73 clinically relevant cancer genes.

Results: A total of 176 somatic activating *RET* alterations were detected in 170 patients (143 fusions and 33 missense mutations). Patients had non–small cell lung (NSCLC, $n = 125$), colorectal ($n = 15$), breast ($n = 8$), thyroid ($n = 8$), or other ($n = 14$) cancers. Alterations in other oncogenic signaling pathway genes were frequently identified in *RET*-positive samples and varied by specific *RET* fusion gene partner. *RET* fusions involving partners other than *KIF5B* were enriched for alterations in MAPK pathway genes and other bona fide oncogenic drivers of NSCLC, particularly *EGFR*. Molecular and clinical data revealed that these variants emerged later in the genomic evolution of the tumor as mechanisms of resistance to EGFR tyrosine kinase inhibitors.

Conclusions: In the largest cancer cohort with somatic activating *RET* alterations, we describe novel co-occurrences of oncogenic signaling pathway aberrations. We find that *KIF5B-RET* fusions are highly specific for NSCLC. In our study, only non-*KIF5B-RET* fusions contributed to anti-*EGFR* therapy resistance. Knowledge of specific *RET* fusion gene partner may have clinical significance.

Introduction

The *RET* proto-oncogene encodes a receptor tyrosine kinase (RTK) and is an emerging target for cancer therapy (1). Next-generation sequencing (NGS) studies have identified *RET* alterations in approximately 2% of diverse solid tumors; however, not all are clearly oncogenic (2, 3). Gain-of-function amino acid substitutions and genomic rearrangements producing chimeric fusion proteins can cause ligand-independent constitutive activation of RET. The most commonly described oncogenic *RET* alterations are in thyroid and non–small cell lung cancers (NSCLC). *RET* rearrangements have been reported in 20%–40% of papillary thyroid cancer (PTC), 1%–2% of NSCLC (particularly in adenocarcinoma histology with minimal tobacco exposure), and occasionally in a variety of other cancer types (4–10). *RET* M918T, a hotspot mutation within the tyrosine kinase domain, is the most common somatic molecular event in sporadic medullary thyroid cancer (MTC), found in 43%–71% of cases, and is associated with poor prognosis (11–13). A subset of patients with MTC have germline *RET* missense mutations. Responses of these tumors to RET inhibition has been reported (14). The prevalence of oncogenic alterations in *RET* is not well described in other cancer types.

Another understudied aspect of *RET*-driven cancers is the extent to which co-occurring genomic alterations exist and how they

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Translational Relevance

Cell-free circulating tumor DNA next-generation sequencing testing may be beneficial at identifying less common, but potentially targetable alterations such as activating *RET* alterations as well as providing an overview of multiple resistance mechanisms that may be present in different tumor populations. This study provides additional evidence to a growing body of literature that advanced stage cancers, particularly those having progressed on targeted therapy, may be driven by multiple oncogenic pathways that may not be apparent from tissue taken at initial cancer diagnosis or from single lesions at the time of progression.

may impact prognosis or therapeutic response. Advanced-stage *EGFR*- or *KRAS*-driven lung cancers commonly harbor co-occurring mutations in pathways that impact tumor biology and/or response to targeted therapy, chemotherapy, or immune checkpoint inhibition (15–19). Tissue NGS compendia often include data on early stage and/or untreated patients, so the landscape of tumors with *RET* aberrations in advanced, treated cancers is not well described. A tissue-based NGS study found that >80% of tumors harboring a *RET* alteration had coexisting alterations, most commonly in *TP53*, cell-cycle–associated genes, the PI3K pathway, MAPK effectors, or other tyrosine kinase families (3). However, this study included variants not clearly known to be oncogenic, germline alterations, and treatment history was largely unavailable. In the setting of treatment-naïve NSCLC, oncogenic drivers, including *RET* fusions, are considered to be mutually exclusive. In contrast, two recent cell-free circulating tumor DNA (cfDNA) studies of patients with NSCLC harboring *EGFR* or *ALK* driver mutations found frequent co-occurrence of multiple oncogenic divers in the setting of acquired resistance to targeted therapy (15, 19). *RET* fusions have been reported as an acquired resistance mechanism to *EGFR* tyrosine kinase inhibitors (TKI) in approximately 5% of osimertinib-resistant biopsies (20–23).

Several FDA-approved multikinase inhibitors (e.g., vandetanib, cabozantinib, lenvatinib, sunitinib, alectinib, sorafenib, ponatinib, nintedanib, regorafenib) have activity against RET and are approved for thyroid cancer, renal cell carcinoma, leukemia, gastrointestinal stromal tumors, colorectal cancer, and/or hepatocellular carcinoma; however, none require identification of a *RET* alteration or other biomarker for patient selection. While these agents are nonspecific, they have been investigated in *RET*-driven NSCLC with response rates as high or higher than with chemotherapy, although lower than that of other oncogene-targeted therapies in NSCLC such as those for *EGFR*, *ALK*, *ROS1*, and *BRAF* V600E (24–26). Gatekeeper *RET* V804M/L mutations may be acquired after targeting of RET fusions with vandetanib or cabozantinib; however, their prevalence in a clinical setting is not known (27–30). More selective RET inhibitors are showing promise in early-phase clinical trials in patients with *RET*-driven advanced solid tumors and also have activity against the V804 gatekeeper mutation (NCT03157128, NCT03037385, NCT01877811; refs. 29, 31–33). There is limited evidence suggesting that specific drugs may have differential anti-RET activity depending on the upstream fusion partner. In a phase II study of vandetinib in advanced NSCLC, 5 of 6 patients with a *CCDC6-RET* fusion achieved an objective response compared with 2 of 10 patients with a *KIF5B-RET* fusion (34). In a phase I/Ib study of the VEGFR-sparing multikinase inhibitor, RXDX-105 in NSCLC, none of the 20 patients with a *KIF5B-RET* fusion responded, whereas 6 of 9 patients with non-*KIF5B RET* fusions responded (35). However, a difference in response rate by fusion partner was not observed in a larger retrospective study evaluating several multikinase inhibitors in NSCLC or in two recent trials evaluating selective RET inhibitors (LOXO-292 and BLU-667) across multiple cancer types (24, 31, 33). Given limited data, the clinical impact of the fusion partner is not clear.

In this study, we evaluated genomic sequencing data from nearly 33,000 patients with diverse advanced cancers tested with a validated comprehensive NGS cfDNA assay, Guardant360 (G360; Guardant Health). We exploit the ability of cfDNA NGS to differentiate germline from somatic *RET* alterations and truncal clonal drivers from acquired subclonal variants (36, 37). cfDNA analysis can provide information on genomic alterations shed from multiple metastatic lesions, thereby capturing tumor heterogeneity that may not be appreciable in the primary tumor or from biopsy of a single metastatic site. We estimate the frequency of *RET* and co-occurring alterations, focusing only on somatic alterations predicted to be functionally relevant.

Materials and Methods

Consecutive patients with at least one somatic *RET*-activating alteration detected on the G360 cfDNA assay were identified from the Guardant Health deidentified database (Guardant Health). Patients were tested between February 2015 and July 2017 and had stage III or IV solid tumors. Only samples with evidence of tumor DNA present (e.g., at least one alteration was present) were included to determine prevalence estimates. Germline alterations were filtered out in this study using a method previously described that differentiates germline from somatic mutations based on the relative variant allele fraction and position of known germline single-nucleotide polymorphisms (36). Patients were included if at least one sample contained a somatic *RET* alteration predicted to be oncogenic, which included *RET* rearrangements and missense alterations leading to single amino acid substitutions that have been previously characterized as causing aberrant *RET* activation. For co-occurring alterations, synonymous alterations and variants of uncertain significance were excluded so that only variants known or predicted to be functionally significant were included. Curation resources include COSMIC, cBioPortal for Cancer Genomics, UniProt, Integrative Genomics Viewer, and literature cataloged by PubMed and the International Agency for Research on Cancer database. Co-occurrence of other alterations was determined on a per patient basis by summarizing all unique alterations present from all samples available for review. Pathway-level prevalence estimates were also determined on a per patient basis, by counting patients with at least one alteration affecting each pathway (patients with multiple alterations in the same pathway were counted once). A list of genes included in each pathway can be found in Supplementary Figs. S3 and S4. Prevalence estimates accounted for the most extensive panel used. Clinical information (cancer type, age at testing, sex, records on treatment history, and/or tissue results) was taken from test request forms and confirmed by the ordering clinician where possible. This research was conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki,

CIOMS, Belmont Report, U.S. Common Rule) with a waiver of patient consent under an Institutional Review Board–approved protocol for the generation of deidentified datasets for research purposes.

G360 is a 68–73-gene CLIA-certified, College of American Pathologists–accredited, New York State Department of Health–approved clinical cfDNA NGS test with analytic and clinical validation reported (37, 38). cfDNA isolation and analytic methods were performed as previously described. Briefly, extracted cfDNA is subjected to paired-end NGS on an Illumnia NextSeq 500 and/or HiSeq 2500 (Illumina, Inc, average read depth 15,000×) following generation of sequencing libraries using nonrandom oligonucleotide adapters and hybrid capture enrichment (IDT, Inc and Aligent Technologies, Inc). Sequencing reads were mapped to the hg19/GRCh37 human reference sequence and were evaluated for SNVs in 68–73 clinically relevant cancer genes as well as small insertions/deletions (indels), gene rearrangements/fusions, and copy number amplification (CNA) in a subset of genes using a proprietary bioinformatics pipeline that reconstructs the original double-stranded cfDNA molecules. The critical regions of *RET* are sequenced (exons 10, 11, 13, 15, and 16; exons 9, 12, and 14 are also sequenced on the 73-gene panel). Fusions known to be biologically important are reported. The reportable range for SNVs, indels, fusions, and CNAs is $\geq$ 0.04%, $\geq$ 0.02%, $\geq$ 0.04%, and $\geq$ 2.12 copies, respectively, with a >99.9999% per-position analytic specificity (38).

In plasma samples from patients with advanced cancer, the majority of cfDNA is typically germline-derived and only a small fraction is tumor-derived. The variant allele fraction (VAF) for a given mutation is the total number of cfDNA molecules harboring the mutation divided by the total number of unique cfDNA molecules at that position. The median VAF using this assay is 0.46% (38). Clonality assessment was based on modeling that considers the relative timing of point mutations and amplifications in a sample and adjusts for mutations on amplified genes as described previously (37). Relative VAF is the copy number–adjusted VAF normalized to the highest VAF in the sample. For the purpose of this study, clonal alterations were those with relative clonality >0.9. The cutoff of >0.9 was chosen as it has been shown to recapitulate mutual exclusivity of truncal oncogenic drivers in NSCLC. Notably, these are likely conservative estimates of clonal alterations as truncal drivers can have clonality <0.9 (37). Comparisons between *RET*-positive and negative cases as well as proportions with co-occurring alterations were performed using two-sided Fisher exact tests. Comparison of median VAFs was done by Kruskal–Wallis test.

Results

Prevalence of activating RET alterations in cfDNA of patients with advanced cancer

Among 32,989 consecutive patients with diverse stage III–IV solid tumors, 176 distinct somatic *RET* alterations predicted to be oncogenic were detected in the cfDNA of 170 (0.5%) patients (patients with multiple samples containing the same *RET* alteration were only counted once). Another 529 patients who had only variants of uncertain significance, inactivating, or synonymous alterations in *RET* were not further evaluated in this study (a list of recurrent *RET* VUS can be found in Supplementary Fig. S1).

Oncogenic *RET* alterations included 143 in-frame fusions found in 141 patients and 33 single-nucleotide variants (SNV) resulting in an amino acid substitution found in 29 patients. *RET* fusions were most prevalent among patients with NSCLC, thyroid cancer, or colorectal cancer (Table 1). Seven different fusion partners (*KIF5B*, *CCDC6*, *NCOA4*, *TRIM24*, *TRIM33*, *ERC1*, *APAF1*) were observed. The most common fusion partner was *KIF5B*, which was only observed in NSCLC ($n = 75$) or cancer of unknown primary (CUP, $n = 2$). Twenty-five different breakpoint combinations were observed, >95% of which involved intron 11 of *RET*, most commonly fused with intron 15 of *KIF5B* or intron 1 of *CCDC6* (Fig. 1A; ref. 39). One patient with colorectal cancer had two *RET* fusions involving different partners (*CCDC6* and *NCOA4*), and one patient with NSCLC had *KIF5B-RET* fusions involving two distinct breakpoints.

RET SNVs were most commonly identified among patients with thyroid or breast cancer, but were found in a range of other cancer types including NSCLC (Table 1; Fig. 1B; Supplementary Table S1). The most common alterations were located in the tyrosine kinase domain ($n = 19$ patients, 8 of whom had M918T) or affected cysteine residues in the extracellular domain ($n = 12$ patients). One patient with gastric cancer had D707N, which affects a caspase cleavage site within the cytoplasmic domain of RET and has been shown to inhibit apoptosis in preclinical studies (40, 41). Two patients had multiple *RET* SNVs. One was a patient with MTC harboring M918T, V804M, and V804L alterations who had been treated with several TKIs in an investigational setting. The other patient had an atypical lung carcinoid harboring C611R, C618Y, and C620F alterations (treatment history unknown).

Co-occurrence with other oncogenic alterations

A total of 210 cfDNA samples were available from the 170 *RET*-positive patients from which to analyze the prevalence of co-occurring alterations and relative clonality. The alterations present in patients with multiple samples were summarized to avoid duplicate counting. Only characterized nonsynonymous alterations known or predicted to have a functionally relevant impact were included (e.g., alterations resulting in enhanced oncogene signaling or loss-of-function mutations in tumor suppressor genes). Patients were evaluated for co-occurring alterations in 72 ($n = 62$ patients), 69 ($n = 75$), or 67 other genes ($n = 33$), based on the assay version available at the time (Supplementary Fig. S2).

A total of 141 patients (82.9%) had 473 additional alterations besides *RET* affecting 52 genes, 291 of which were unique variants. The prevalence of co-occurring alterations was similar between samples that had a *RET* fusion and those with a *RET* SNVs. The median number of additional alterations per patient was 3 (range 1–25), including copy number amplifications (CNA). The most common alterations were in *TP53* ($n = 99/170$ patients, 58.2%), other receptor tyrosine kinases (RTK, $n = 57/170$, 33.5%), or genes involved in the cell cycle ($n = 48/170$, 28.2%), MAPK ($n = 46/170$, 27.1%), and PI3K ($n = 27/170$, 15.9%) pathways (Supplementary Fig. S3). The proportion of patients with at least one co-occurring alteration affecting various oncogenic pathways differed on the basis of the *RET* alteration type (Fig. 2).

Other RTK alterations co-occurred with *RET* mutations only in patients with CUP, colorectal cancer, or NSCLC. In contrast to previous tissue-based studies, *RET* fusions were found in samples positive for other driver mutations in NSCLC and/or with MAPK pathway alterations (3). Alterations in at least one MAPK pathway

Table 1. Prevalence of oncogenic *RET* alterations detected in the cfDNA of patients with advanced solid tumors, by tumor type, and *RET* alteration type (including subcategories of *RET* fusion or SNVs)

Alteration, number of unique patients (%)	NSCLC (*n* = 14,639)	Colorectal (*n* = 3,059)	Breast (*n* = 3,921)	Thyroid (*n* = 137)	Other[a] (*n* = 11,233)	Total (*n* = 32,989)
Patients with any *RET* fusion	116 (0.79)	14 (0.46)[b]	2 (0.05)	1 (0.73)	8 (0.07)	141 (0.4)
KIF5B-RET	75	0	0	0	2[c]	77
CCDC6-RET	31	6	2	1	3	43
NCOA4-RET	6	8	0	0	0	14
Other[d]	4	1	0	0	3	8
Patients with any RET SNV	9 (0.06)	1 (0.03)	6 (0.15)	7 (5.1)[e]	6 (0.05)[f]	29 (0.09)
M918T	0	0	3	5	0	8
Other TKD	5	0	1	3	2	11
CRD	4	1	2	1	3	13
Other	0	0	0	0	1	1

Abbreviations: CRD, cysteine-rich domain; TKD, tyrosine kinase domain.

[a]Other cancer types with RET fusion included CUP (*n* = 4), small-cell lung (*n* = 1), esophageal (*n* = 1), head and neck (*n* = 1), mesothelioma (*n* = 1); or with RET SNV included atypical pulmonary carcinoid (*n* = 1), gastric (*n* = 1), bladder (*n* = 1), prostate (*n* = 1), pancreatic neuroendocrine (*n* = 1), and sarcoma (*n* = 1).

[b]One patient with colorectal cancer had two *RET* fusions (*CCDC6-RET* + *NCOA4-RET*).

[c]Both patients with *KIF5B-RET* fusions without NSCLC had unknown primary cancers.

[d]Other fusion partners included *ERC1* (*n* = 3), *TRIM24* (*n* = 2), *TRIM33* (*n* = 2), and *AFAP1* (*n* = 1).

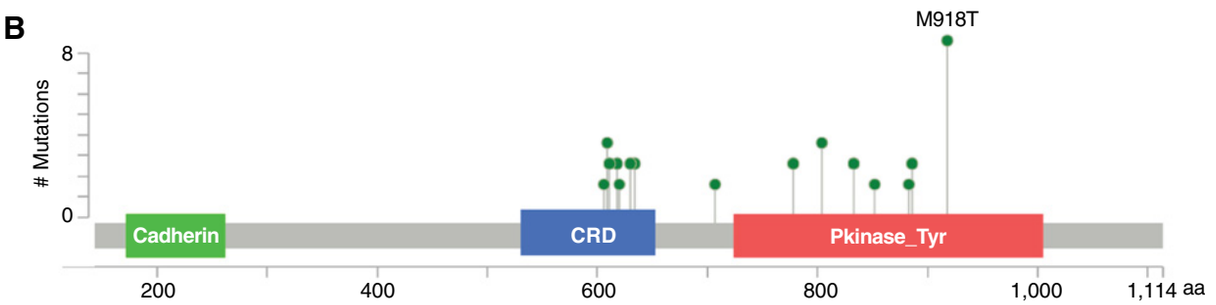
[e]One patient with MTC had 3 TKD alts.

[f]One patient with atypical pulmonary carcinoid had 3 CRD alts.

A

RET Intron	*KIF5B* Intron			*CCDC6* Intron		*NCOA4* Intron			Other
	15	16	Other	1	Other	7	8	10	Other
9	–	–	1	–	–	–	–	–	–
10	–	–	1	3	–	–	–	–	–
11	61	6	9	36	4	1	5	8	8
12	–	–	1	–	–	–	–	–	–

B



Number of patients with alterations by domain		
Cysteine-rich	Tyrosine kinase	Other
Y606C (*n* = 1)	V778I (*n* = 2)	D707N (*n* = 1)
C609F/Y (*n* = 3)	V804L/M (*n* = 3)	
C611R/Y (*n* = 2)	R833C (*n* = 2)	
C618Y (*n* = 2)	I852M (*n* = 1)	
C620F (*n* = 1)	R886W (*n* = 2)	
C630R (*n* = 2)	A883F (*n* = 1)	
C634G/R (*n* = 2)	M918T (*n* = 8)	
Total	*13*	*19*	*1*

Figure 1.

Somatic oncogenic *RET* alterations detected by NGS of cfDNA from patients with advanced solid tumors. **A,** Distribution of breakpoints detected among samples containing *RET* rearrangements (*n* = 24 unique fusions involving 7 different gene partners in 156 samples from 141 patients). **B,** Distribution of characterized SNVs in the coding region of *RET* (*n* = 19 unique alterations in 34 samples from 29 patients). Counts are unique patients. CRD, cysteine-rich domain.

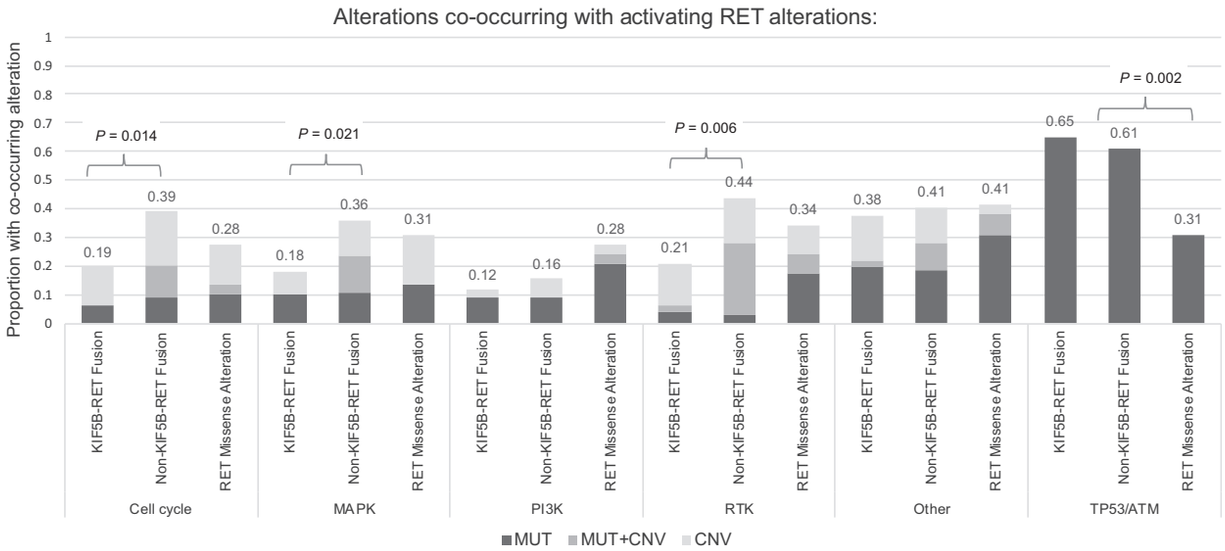


Figure 2.

Co-occurring somatic genomic alterations detected in the cfDNA of 170 patients with advanced solid tumors harboring an activating *RET* mutation, by pathway and *RET* alteration type (*KIF5B-RET* fusion, fusion involving partner other than *KIF5B*, and *RET* missense SNVs). Proportions are calculated by counting the number of patients with at least one sample containing an alteration of one or more genes in the pathway (see also Supplementary Fig. S3).

gene were observed with *RET* fusions in 37 of 141 (26.2%) patients with either breast cancer, colorectal cancer, NSCLC, or CUP.

Given the cfDNA testing population is primarily comprised of patients with advanced and typically pretreated cancers, we hypothesized this novel observation was due to selection of *RET* and/or MAPK pathway alterations as a mechanism of therapy resistance. Across all *RET*-positive samples, approximately 30% of the 197 oncogenic *RET* and 2% of the 42 non-CNA MAPK pathway alterations detected were clonal (clonality >0.9) based on a validated copy number–adjusted clonality assessment (see Methods). This suggested that a proportion of *RET* variants and the majority of the MAPK pathway variants detected in this series emerged later in the genomic evolution of the tumor. We next examined the contribution of *RET* as a driver or resistance mutation by evaluating relative clonality and patterns of co-occurring alterations. We focused on NSCLC for which the most genomic and clinical data were available and because patterns of therapy resistance are relatively well-described.

RET fusions in NSCLC may arise as a mechanism of resistance to EGFR tyrosine kinase inhibitors

Among the 125 *RET*-positive patients with NSCLC, 103 (82.4%) had at least one additional functionally relevant somatic alteration (Fig. 3A). Twenty-three patients had another classic driver mutation detected in at least one cfDNA sample though the proportion differed significantly by *RET* mutation type (Fig. 3B). Only two of the 75 (2.7%) patients with a *KIF5B-RET* fusion had other NSCLC drivers including one with *KRAS* K117N and one with *EGFR* R776H, which has been shown to confer constitutive ligand-independent activation of *EGFR in vitro* but is not well-described in patients (42, 43). In both cases, the *KIF5B-RET* fusion had higher clonality (1 vs. 0.47 in the *KRAS*-positive case and 0.54 vs. 0.05 in the *EGFR*-positive case), suggesting that the *RET* fusion was likely a primary driver in both cases. We also cannot rule out the possibility that the *RET/KRAS*–positive case could have had an additional primary cancer or clonal hematopoiesis given a lack of detailed clinical history. Overall the median clonality of *KIF5B-RET* fusions in NSCLC was 0.55 (95% CI: 0.47–0.8) and in all *KIF5B-RET*–positive samples, the mutations that did have higher clonality were in tumor suppressor genes or were VUS or synonymous alterations (Fig. 3C). These observations are consistent with the *KIF5B-RET* fusions being a primary NSCLC driver.

In contrast, other NSCLC driver mutations were commonly observed in samples containing *RET* SNVs and *RET* fusions involving partners other than *KIF5B*. Among the 9 patients with NSCLC and *RET* SNVs, 4 (44%) had classic driver mutations, including two with *KRAS* alterations, 1 with an *EGFR* exon 20 insertion, and 1 with an *ERBB2* exon 20 insertion. Median clonality of *RET* SNVs was similar in samples containing another driver mutation as those without another driver (0.47 vs. 0.41, respectively; Fig. 3C). The *RET* SNVs in patients with NSCLC affected codon 609 ($n = 2$), 778 ($n = 2$), 630, 833, 886, 606, and 804 ($n = 1$ each). Cumulatively, our data are inconclusive as to whether *RET* SNVs have a strong role as drivers of NSCLC.

Seventeen of the 41 (41.4%) patients with a non-*KIF5B RET* fusion had one or more samples positive for either a deletion of *EGFR* exon 19 ($n = 13$) or an L858R mutation ($n = 2$) or a *KRAS* G12V or K117N alteration ($n = 1$ each). The median clonality of the non-*KIF5B-RET* fusion among *EGFR*-positive samples was significantly lower than those without *EGFR* mutations (0.08 vs. 0.53, $P<0.0001$; Fig. 3C). Consistent with this observation, the *RET* fusion appeared to be arising as a mechanism of resistance to EGFR TKIs in all 15 patients (Fig. 4). Twelve of the 15 (80%) *RET/EGFR*$^+$ cases also had the *EGFR* T790 gatekeeper mutation detected, three of whom also had *EGFR* C797S, which has been shown to arise as a mechanism of resistance to osimertinib (44). *CTNNB1* exon 3 alterations were found in 3 of the 15 patients. While *CTNNB1*

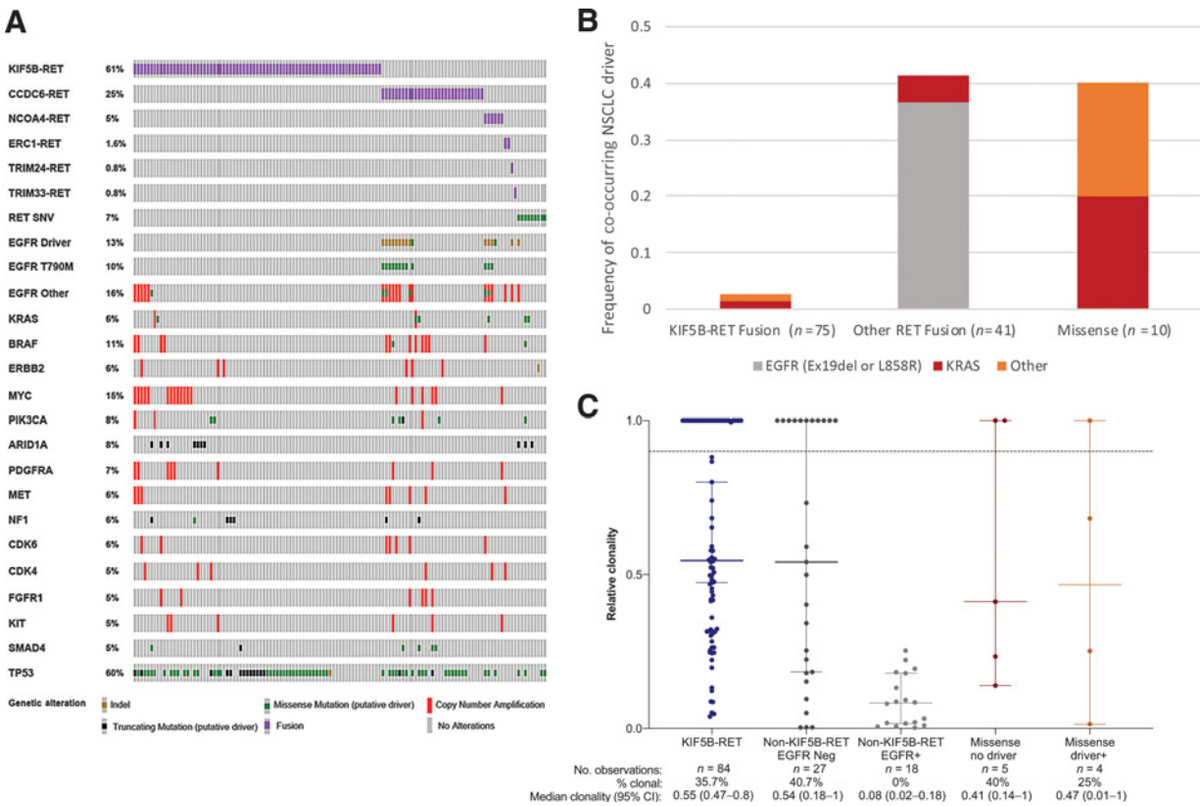


Figure 3.

Co-occurring somatic alterations detected by NGS of cfDNA from 125 patients with advanced NSCLC harboring an oncogenic *RET* mutation. **A,** Oncoprint of somatic genomic alterations present in at least 5% of samples. The first four rows indicate *RET* rearrangements by fusion partner, and the fifth row represents *RET* oncogenic SNVs. **B,** Co-occurrence of oncogenic *RET* mutations with mutations in classic NSCLC driver genes: *EGFR* exon 19 deletion or L858R (gray), *KRAS* (red), or other (orange). Other includes *EGFR* R776H, *EGFR* exon 20 insertion, *ERBB2* exon 20 insertion ($n = 1$ patient each). One patient with *EGFR*-positive NSCLC also had an *ALK* rearrangement and another had *BRAF* V600E in the setting of *EGFR* TKI resistance (see Fig. 4). No cases of *ROS1* rearrangements, *MET* exon 14 skipping, or *ERBB2*-activating SNVs were observed. **C,** Relative clonality of oncogenic *RET* alterations (see Methods). Solid horizontal lines indicate median and 95% confidence intervals, and dashed line indicates clonality >0.9. Medians and proportions are calculated on a per alteration basis across all samples.

mutations are enriched in *EGFR*-mutant NSCLC, particularly clones that have acquired T790M, this frequency is higher than what has been reported in other *EGFR* T790M-positive NSCLC cfDNA cohorts (15). In total, 14 of 15 *RET*/*EGFR*–positive patients' tumors harbored at least one, and typically multiple, known genomic mechanisms of resistance to *EGFR* TKIs (45). While serial sampling and pre-EGFR TKI tissue *RET* status were not available for most of the cases to confirm whether *RET* was acquired over time, the copy number–adjusted *EGFR* driver to *RET* fusion variant allele fraction (VAF) ratio was consistent with the *RET* fusion arising later in tumorigenesis in all 15 cases (Fig. 5A).

Furthermore, 11 of the 15 patients were indicated on test request forms as having prior exposure to *EGFR* TKIs and more detailed treatment and molecular testing information was available for patients GH-001 to GH-006 (Fig. 5B). These 6 patients all had lung adenocarcinoma at a median age of diagnosis of 55 years (range 41–65). Five had *EGFR* exon 19 deletion and one had L858R detected in tissue either prior to first-line therapy (patients GH-002-GH-006) or shortly after starting first-line chemotherapy (patient GH-001). Only one patient (GH-006) had evaluation of *RET* in tissue collected prior to *EGFR* TKI exposure that was *RET* negative by NGS. All 6 patients had been treated with at least one *EGFR* TKI prior to collection of the cfDNA assay that detected the *RET* fusion. The median time on TKI prior to detection of the *RET* fusion in cfDNA was 17.5 months (range 6–46 months). The *RET* fusion was detected following therapy with erlotinib in 3 patients, and osimertinib and first- or second-generation TKIs in the other 3 patients. The *RET* fusion was found without ($n = 1$), concurrent with ($n = 2$), and subsequent to ($n = 3$) the first detection of *EGFR* T790M. Three patients (patients GH-001, GH-002, and GH-003) had multiple cfDNA NGS assays. In two patients (GH-002 and GH-003), *EGFR* T790M, but not the *RET* fusion, was detected on the first sample that was drawn following progression on erlotinib and/or afatinib. The *RET* fusion was detected on a subsequent sample following progression on osimertinib. These cases are consistent with the *RET* fusion arising as an acquired mechanism of resistance.

Patients GH-002, GH-004, and GH-005 died shortly after the detection of the *RET* fusion without a change in therapy. Patient GH-001 had tissue "hotspot" testing for T790M that was negative upon progression after 46 months of treatment with erlotinib. The patient was placed on nivolumab and had stabilization of disease.

Patient	Sample	*RET* fusion partner (VAF)	*EGFR* Alterations (VAF)					Other EGFR TKI Resistance Alterations (VAF)	Copy number amplifications													Prior EGFR TKI	Time on EGFR TKI (mo)	Time from dx (mo)
			Exon 19del	L858R	T790M	C797S	Other		*AR*	*BRAF*	*CCND1*	*CCND2*	*CCNE1*	*CDK4*	*CDK6*	*EGFR*	*ERBB2*	*FGFR1*	*KIT*	*MET*	*MYC*	*PDGFRA*			
GH-001	1	*CCDC6* (0.1)	22.9		4.7		T854A (3.9)																E	46	52
	2	ND																					E	46	66
GH-002	1	*CCDC6* (0.2)	15.4		10			*BRAF* V600E (0.9) *PIK3CA* E542K (0.1)															E,A,O,G	37	60
GH-003	1	ND	0.2		0.2																		A	15	15
	2	*CCDC6* (0.1)	0.7					*PIK3CA* G118D (.7)															A,O	21	21
	3	*CCDC6* (0.4)	1.5					*PIK3CA* G118D (1.0)															A,O	27	27
GH-004	1	*NCOA4* (0.4)		3.1																			E	14	20
GH-005	1	*NCOA4* (0.4)	61.5		14.2	1.4 0.4		*KRAS* G12S (0.3) *NRAS* Q61H (0.2) *RB1* p.Asp270fs (62.9)															E,O	14	19
GH-006	1	*CCDC6* (0.6)	77		19.7			*STRN-ALK* Fusion (0.2)															E	6	6
GH-007	1	*CCDC6* (3.3)	24.6				G724S (0.2)	*CTNNB1* S45P (15.3)															E	NA	NA
GH-008	1	ND	22.3		7.6			*CTNNB1* S33C (.9) *CTNNB1* S33P (.3)															E	NA	NA
	2	*CCDC6* (0.1)	1.8					*CTNNB1* S33C (.1)															O	NA	NA
GH-009	1	*TRIM24* (4.7)	37.4					*RB1* p.Arg7fs (9.6)															O	NA	NA
GH-010	1	*CCDC6* (0.3)	3.7		1.9			*CTNNB1* S37F (1.2)															O	NA	NA
	2	*CCDC6* (0.2)	2.6		0.8			*CTNNB1* S37F (.6)															O	NA	NA
	3	*CCDC6* (1.1)	12.4		4.7			*CTNNB1* S37F (3.8)															O	NA	NA
GH-011	1	*CCDC6* (1.3)		46.5	16.8																		E	NA	NA
GH-012	1	ND	1.1		0.6																		E	NA	NA
	2	*NCOA4* (1.3)	9.2																				NA	NA	NA
GH-013	1	*CCDC6* (0.1)	8.1		6.4			*PIK3CA* H1047R (1.7)															NA	NA	NA
GH-014	1	*NCOA4* (0.2)	38.4		48.7	12.6 11.3	L792F (0.3)																NA	NA	NA
GH-015	1	*CCDC6* (0.5)	19.4		7.7	2.9																	NA	NA	NA

Figure 4.

Landscape of genomic alterations found in the cfDNA of 15 patients with both an *EGFR* driver mutation (exon 19 deletion or L858R) and a *RET* fusion. The variant allele frequency (VAF) of detected alterations in each of the 22 samples from the 15 patients is shown for fusions, SNVs, and indels. Samples with copy number amplification are shaded gray. Only select alterations that are known mechanisms of resistance to anti-*EGFR* therapy are shown for readability. Common tumor suppressor alterations (e.g., NF1, TP53) and other SNVs (BRCA2 C3155S in patient 5, and MLH1 E433Q and JAK2 V617F in patient 12) are not shown. Patients GH-001, GH-003, GH-008, GH-010, and GH-012 had multiple samples collected; in some, the *RET* rearrangement was not detected in all samples. Exposure to *EGFR* tyrosine kinase inhibitors (TKI) prior to sample collection is indicated in the last three columns. Patients GH-001 through GH-006 had detailed history available, including order of therapies, duration of therapy in months (mo), and other molecular testing history (see Fig. 5B). Treatment history of the other patients was taken from test request forms when available. A, afatinib; E, erlotinib; G, gefitinib; NA, not available; ND, not detected; O, osimertinib.

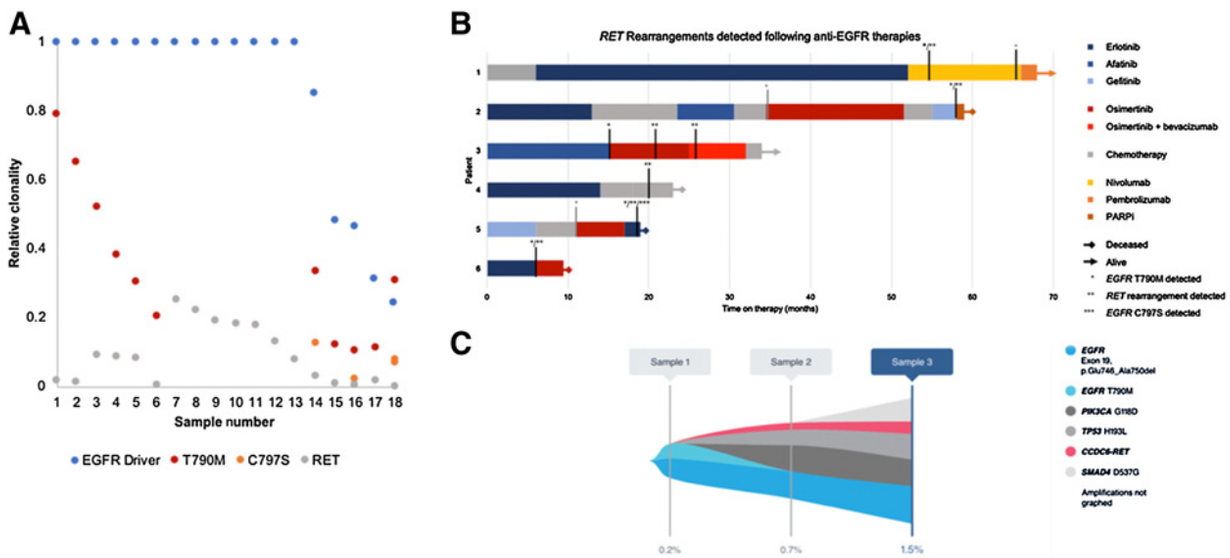


Figure 5.

Landscape of genomic alterations found in the cfDNA of 15 patients with both an *EGFR* driver mutation (exon 19 deletion or L858R) and a *RET* fusion. **A,** Relative clonality of the *EGFR* driver mutation and *RET* fusion compared with on-target *EGFR* TKI mechanisms of resistance (T790M and C797S) among the 18 samples with *RET* fusion detected. **B,** Order and duration of systemic therapies among the 6 patients with both an *EGFR* driver mutation (exon 19 deletion or L858R) and a *RET* rearrangement detected in cfDNA and detailed clinical history available. *EGFR* TKIs are shown in shades of blue or red, chemotherapy (various) in gray, and other therapies in orange. Patients still living are indicated with an arrow, and time of death is indicated with a diamond. Vertical black lines indicate timing of the Guardant360 blood draw relative to treatment timing, and vertical gray lines indicate timing of other liquid biopsy or tissue molecular tests. The first detection of *EGFR* T790, the *RET* fusion, or *EGFR* C797S is indicated with a single, double, or triple asterisk, respectively. **C,** Tumor response map for patient GH-003 showing suppression of *EGFR* T790M between sample 1 and sample 2 while the patient was on osimertinib, but emergence of *RET* fusion and alterations in PIK3CA and TP53. Treatment between samples 2 and 3 included addition of bevacizumab to osimertinib with relatively stable VAF of the *RET* fusion, PIK3CA, and TP53 alterations, but emergence of a SMAD4 alteration.

Two months after initiating nivolumab, the patient had G360 testing that revealed 5 *EGFR* alterations (exon 19 deletion, T790M, L777M, T854A, and amplification), a *CCDC6-RET* fusion and amplifications of *MET*, *BRAF*, *AR*, and *CDK6*. The patient continued on nivolumab and another G360 was drawn after 12 months that showed resolution of the *EGFR* and *RET* alterations. The patient was switched to pembrolizumab due to side effects and continues to have stable disease 2 months after starting pembrolizumab.

In patient GH-003, G360 revealed *EGFR* exon 19 deletion and T790M following progression on afatinib (15-month treatment duration). Therapy was switched to osimertinib; however, after 7 months, the patient experienced disease progression. A second G360 at that timepoint identified the *EGFR* exon 19 deletion, the *RET* fusion as well as alterations in *TP53* and *PIK3CA*, but not *EGFR* T790M, implicating the *RET* fusion as a mechanism of resistance independent of *EGFR* T790M (Fig. 5C). The patient was continued on osimertinib for another 10 months with the addition of bevacizumab. On progression, a third G360 revealed the same *EGFR* exon 19 deletion, the *RET* fusion, *TP53*, and *PIK3CA* alterations as well as a new alteration in *SMAD4*; however, *EGFR* T790M was not detected. The patient was placed on chemotherapy and had a partial response according to RECIST 1.1 criteria.

In patient GH-006, following progression on erlotinib, a G360 test revealed an exon 19 deletion, T790M, and *EGFR* amplification in addition to the *RET* fusion, an *ALK* rearrangement, a *TP53* alteration, and amplifications of *MYC* and *CDK6*. Therapy was switched to osimertinib. The patient had a mixed response, progressed after 3.5 months of therapy, and died shortly thereafter.

Across the entire dataset, the prevalence of *RET* fusions among all patients with NSCLC with an *EGFR* exon 19 deletion was higher than that of patients with L858R (0.8% vs. 0.2%, $P = 0.04$) and higher among patients with coexisting T790M and/or C797S versus those with *EGFR* drivers without T790M or C797S (1.1% and 4.6%, respectively, vs. 0.6%). Among patients with NSCLC with treatment history information submitted at the time of sample collection, the prevalence of a *RET* fusion was higher following exposure to osimertinib (9/184 samples, 4.9%) than first- or second-generation *EGFR* TKIs (13/1,627 samples, 0.8%, $P = 0.0001$).

Discussion

This is the largest series to date describing the genomic features of advanced cancers harboring activating somatic *RET* alterations. Somatic activating *RET* alterations are found in cfDNA of approximately 1 in 200 patients in a wide range of solid tumor types. They are potentially targeteable as demonstrated by the successful treatment of cfDNA NGS-detected *RET* fusion driver alterations in NSCLC (25). In nearly 33,000 patients, *KIF5B-RET* fusions were only identified in patients with NSCLC ($n = 75$) or CUP ($n = 2$), suggesting that the presence of a *KIF5B-RET* fusion may be pathognomonic for a NSCLC diagnosis. If confirmed in a larger CUP cohort, this finding may help determine patients with CUP with NSCLC primary tumors.

The detection of both clonal and acquired subclonal *RET* alterations, including notation of the fusion partner for rearrangements, builds upon previous findings that a plasma-based cfDNA NGS assay may be useful. The quantitative nature of variant allele fractions (VAF) in cfDNA permits the assessment of the relative burden of various alterations contributing to tumor progression (15, 37). The highest VAF alterations are generally the earliest events in tumorigenesis while alterations that arise later in tumorigenesis tend to have a VAF that is lower than these truncal events.

In a study of 4,871 diverse tumor types tested with a 182- or 236-gene tissue NGS panel, 88 (1.3%) had a *RET* alteration and similar to this study, most (81%) had coexisting alterations (although all here are non-VUS alterations; ref. 3). In tissue, *RET* fusions did not co-occur with alterations in MAPK effectors and co-occurred with alterations in other tyrosine kinase family genes in only 7.4% of cases. In contrast, in this cfDNA cohort, *RET* fusions co-occurred with alterations in these pathways in 26.2% and 33.3% of patients, respectively.

Given that the cfDNA assay used in this study has been extensively validated with high specificity, the differences in co-occurring alterations between plasma and tissue are likely due to biological factors and we provide evidence that this observation is likely a result of some *RET* fusions and MAPK pathway alterations arising as a mechanism of therapy resistance (38). The cfDNA testing population is comprised of patients with advanced cancers and who are often treated with one or more systemic therapies prior to sample collection, whereas tissue compendia may contain early-stage tumors that have not been exposed to systemic therapy. Furthermore, cfDNA overcomes challenges of intra- and inter-tumor heterogeneity. The frequent presence of subclonal resistance alterations has previously been shown to account for major differences in molecular profiles identified using cfDNA NGS in advanced generally pretreated cohorts compared with generally early-stage, treatment-naïve tissue cohorts (37). The differences between tissue and cfDNA genomic landscapes highlight the value of reassessing tumor genomic status in patients with advanced cancer, particularly following treatment with a targeted agent. As activation of *RET* promotes downstream pathways including RAS/MAPK, JAK/STAT, and PI3K/AKT, inhibiting *RET* in the setting of a co-occurring MAPK or other downstream pathway may be less efficacious (4, 46, 47).

The majority of cfDNA-detected MAPK and other RTK alterations were identified in patients with NSCLC or colorectal cancer who may have received EGFR-directed therapy prior to cfDNA collection and acquired alterations in these pathways as a mechanism of therapy resistance (48, 49). Indeed, NSCLCs harboring subclonal *RET* fusions and coexisting *EGFR* driver mutations with available treatment histories all had exposure to first-, second-, and/or third-generation *EGFR* TKIs prior to cfDNA sample collection. Additional work in a larger cohort is needed to better understand the contribution of *RET* to therapy resistance or as a driver in other cancer types, particularly in colorectal cancer and breast cancer that are commonly treated with targeted therapies.

Interestingly, only acquired subclonal non-*KIF5B-RET* fusions (particularly with *CCDC6* or *NCOA4* as the fusion partner) arise at progression in NSCLC, whereas the more common gene partner is *KIF5B* when *RET* fusions are the clonal oncogenic driver. The different frequency of fusion partners seen as drivers versus acquired mechanisms of resistance in NSCLC has been reported (19). The dominant partner in *ALK* fusion drivers is *EML4* (>95% of cases), whereas *EML4* is the partner in only approximately 45% of *EGFR* TKI–resistant cases. However, the fact that no *KIF5B-RET* fusion arose as a mechanism of resistance in this series suggests emergent *KIF5B-RET* fusions may not be

tolerated in the presence of anti-EGFR TKI and/or that loss of *CCDC6* or *NCOA4* affords a particular survival advantage to tumors exposed to an EGFR TKI. In a *Drosophil*a model, KIF5B-RET fusions (particularly the motor domain that is unique to *KIF5B* relative to the other fusion partners) are highly reliant on EGFR signaling to promote enhanced cell growth, more so than *CCDC6-RET* or NCOA4-*RET* fusions (50). There may be important cell biology differences based on the *RET* fusion partner. Future studies of *RET* should involve assays such as NGS that can distinguish between fusion partners.

While non-*KIF5B-RET* fusions appear to be a rare mechanism of *EGFR* TKI resistance, they may be more likely to emerge in more advanced disease given their frequent co-occurrence with multiple mechanisms of *EGFR* TKI resistance. Our data also suggest that acquired *RET* fusions may be more common following exposure to osimertinib than to first- or second-generation EGFR TKIs. While our observation that 4.9% of NSCLCs with prior osimertinib exposure had a *RET* fusion should be interpreted with caution given the limited available clinical history, Piotrowska and colleagues recently found *RET* fusions in a similar proportion (2/41, 4.9%) of confirmed osimertinib-resistant biopsies (23). The cfDNA results of patient GH-003 suggest that during therapy with osimertinib, the *RET* fusion arose concurrent with suppression of T790M indicating that *RET* fusions may arise in subclones separate from other mechanisms of resistance. Piotrowska and colleagues reported two patients with similar findings where the T790M resistance clone was successfully suppressed with osimertinib while the *RET* fusion was detected at progression. In these two cases, combination therapy with osimertinib and BLU-667 (a novel selective RET inhibitor) was well-tolerated and led to rapid radiographic responses. Given the recent FDA approval of osimertinib for first-line treatment of *EGFR*-driven NSCLC, *RET* fusions may be a relevant factor for treatment selection at progression. The frequency and diversity of coexisting altered oncogenic pathways suggests that comprehensive genomic profiling may be needed in patients progressing on EGFR TKIs to appreciate the entire resistance profile and that optimal targeting of patients with acquired *RET* alterations may require customized combination strategies. Detection of targetable resistance mutations using a comprehensive cfDNA assay may facilitate enrollment in clinical trials as well as serve as biomarkers for therapeutic efficacy.

This study is a retrospective review of genomic findings from a cohort of patients with clinically ordered cfDNA testing and complete treatment history and follow-up data are not available for most of the cohort. Even with this limitation, this cohort offers a snapshot of advanced cancer genomics encountered in a "real-world" setting. As patients for this study were selected on the basis of having a positive *RET* alteration in cfDNA, the overall sensitivity of plasma detection of *RET* alterations cannot be assessed. In addition, the cfDNA assay used only reports *RET* fusions with partners known to be biologically significant. However, as has been previously reported by Zill and colleagues, we found the prevalence of intronic breakpoints largely recapitulates what has been described in tissue, which provides validation of the cfDNA NGS assay from a biological perspective (37).

In summary, cfDNA NGS testing may be beneficial at identifying less common, but potentially targetable alterations in *RET* as well as multiple resistance mechanisms that may be present in different tumor populations. This study provides additional evidence that advanced-stage cancers, particularly those having progressed on targeted therapy, may be driven by multiple oncogenic pathways, which may not be apparent from tissue taken at initial diagnosis. The clinical impact of these findings, such as targeting acquired *RET* fusions in NSCLCs resistant to anti-*EGFR* therapy, requires further investigation. As cfDNA testing provides a noninvasive method of capturing tumor heterogeneity and can evaluate the dynamics of response to therapy, it may provide a useful platform for further studying the impact of clonal and subclonal mutations on tumor progression as well as inform studies of rational combination therapies.

Disclosure of Potential Conflicts of Interest

T.A. Rich has ownership interests (including patents) at Guardant Health. K. L. Reckamp is a consultant/advisory board member for and reports receiving commercial research support from Guardant. Y.K. Chae reports receiving speakers bureau honoraria from Genentech, AstraZeneca, Guardant Health, and Lilly Oncology; is a consultant/advisory board member for Foundation Medicine, Guardant Health, Takeda, Genentech, and AstraZeneca; and reports receiving commercial research grants from Biodesix, Freenome, Lexent Bio, Abbvie, and Bristol-Myers Squibb. R.C. Doebele has ownership interests (including patents) at Rain Therapeutics; is a consultant/advisory board member for Rain Therapeutics, Genentech/Roche, Bayer, and Takeda/Millenium; and has licensing fees for a patent with Abbott. W.T. Iams is a consultant/advisory board member for Outcome Insights and has done clinical trial planning conference travel for EMD Serono. R.B. Lanman is an employee of Biolase; has ownership interests (including patents) at Guardant Health, Biolase, and Forward Medical; and is a consultant/advisory board member for Forward Medical. J.W. Reiss is a consultant/advisory board member for Loxo Oncology. T.E. Stinchcombe is a consultant/advisory board member for Takeda, AstraZeneca, Genetech/Roche and G1 Therapeutics. V. Subbiah reports receiving commercial research support from LOXO Oncology, Blueprint Medicines, Exelixis, Takeda, Roche, Novartis, Bayer, GlaxoSmithKline, Nanocarrier, Vegenics, Celgene, Northwest Biotherapeutics, Berghealth, Incyte, Fujifilm, Pharmamar, D3, Pfizer, Multivir, Amgen, Abbvie, Alfasigma, Agensys, Boston Biomedical, Idera Pharma, and Inhibrx. S.R. Fairclough has ownership interests (including patents) at Guardant Health. J. Yen has ownership interests (including patents) at Guardant Health. No potential conflicts of interest were disclosed by the other authors.

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