Clinical Cancer Research

Clinical Utility of Cell-Free DNA for the Detection of *ALK* Fusions and Genomic Mechanisms of ALK Inhibitor Resistance in Non–Small Cell Lung Cancer

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Abstract

Purpose: Patients with advanced non–small cell lung cancer (NSCLC) whose tumors harbor anaplastic lymphoma kinase (*ALK)* gene fusions benefit from treatment with ALK inhibitors (ALKi). Analysis of cell-free circulating tumor DNA (cfDNA) may provide a noninvasive way to identify *ALK* fusions and actionable resistance mechanisms without an invasive biopsy.

Patients and Methods: The Guardant360 (G360; Guardant Health) deidentified database of NSCLC cases was queried to identify 88 consecutive patients with 96 plasma-detected *ALK* fusions. G360 is a clinical cfDNA next-generation sequencing (NGS) test that detects point mutations, select copy number gains, fusions, insertions, and deletions in plasma.

Results: Identified fusion partners included *EML4* (85.4%), *STRN* (6%), and *KCNQ*, *KLC1*, *KIF5B*, *PPM1B*, and *TGF* (totaling 8.3%). Forty-two *ALK*-positive patients had no history of targeted therapy (cohort 1), with tissue *ALK* molecular testing attempted in 21 (5 negative, 5 positive, and 11 tissue insufficient). Follow-up of 3 of the 5 tissue-negative patients showed responses to ALKi. Thirty-one patients were tested at known or presumed ALKi progression (cohort 2); 16 samples (53%) contained 1 to 3 *ALK* resistance mutations. In 13 patients, clinical status was unknown (cohort 3), and no resistance mutations or bypass pathways were identified. In 6 patients with known *EGFR*-activating mutations, an *ALK* fusion was identified on progression (cohort 4; 4 *STRN*, 1 *EML4;* one both *STRN* and *EML4*); five harbored *EGFR* T790M.

Conclusions: In this cohort of cfDNA-detected *ALK* fusions, we demonstrate that comprehensive cfDNA NGS provides a noninvasive means of detecting targetable alterations and characterizing resistance mechanisms on progression. *Clin Cancer Res; 24(12); 2758–70. ©2018 AACR.*

Introduction

The identification and targeting of oncogenic drivers such as anaplastic lymphoma kinase (*ALK*), epidermal growth factor receptor (*EGFR*), and v-ros1 (*ROS1*) have had a dramatic impact on the treatment of advanced non–small cell lung cancer (NSCLC; refs. 1–4). Patients whose tumors harbor *ALK* gene fusions demonstrate significant clinical benefit from treatment with ALK inhibitors (ALKi); however, their cancer ultimately progresses (5–8). Repeat tumor biopsy on progression has been helpful in determining the optimal subsequent line of treatment in patients receiving oncogene-targeted therapy and is now recommended in the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines for patients with *EGFR*-mutant NSCLC (9). Multiple next-generation ALKi are approved by the FDA but have differential sensitivity profiles with respect to *ALK* kinase domain mutations, suggesting that genomic reprofiling after failure of first-line ALKi may have a role for patients with *ALK*-mutant NSCLC as well (10, 11).

Sampling progressing tumor lesion(s) to identify resistance mechanisms is the standard strategy to evaluate mechanisms of drug resistance; however, this is often complicated by tumors that are inaccessible to biopsy or by tissue that is of insufficient quantity or quality to perform molecular testing. As many as 25% of tumor biopsies are inadequate or insufficient for molecular analysis (12). Additionally, sampling of a single lesion may not provide an accurate representation of the tumor genomic landscape due to tumor heterogeneity (13–15). Finally, rebiopsy presents a small risk of serious complications such as pneumothorax, bleeding, or infection (16, 17).

Next-generation sequencing (NGS) of cell-free circulating tumor DNA (cfDNA) can provide a noninvasive method of

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Translational Relevance

The successful treatment of patients with *ALK*-positive non–small cell lung cancer and identification of resistance mechanisms to targeted therapy are predicated on identifying genetic alterations in tumor cells. However, tumor tissue is not always available. Our data demonstrate that comprehensive cfDNA NGS testing can often noninvasively detect targetable alterations in newly diagnosed patients as well as resistance mutations and possible bypass pathways in patients with progression on targeted therapy. Additionally, we demonstrate the utility of cfDNA to provide a comprehensive view of the diversity and complexity of resistance mechanisms in a heterogeneous tumor cell population.

obtaining and evaluating tumor DNA in patients with cancer. cfDNA-based diagnostics can identify oncogenes at initial diagnosis when tissue samples are insufficient or unobtainable, which can guide effective first-line therapy and can identify actionable resistance mechanisms at disease progression. Prior work has demonstrated the feasibility of using "hotspot" and comprehensive cfDNA profiling for identification of activating mutations and therapeutic resistance mechanisms in *EGFR*-mutated NSCLC (18–24). However, its utility in evaluating *ALK* gene fusions has not been assessed on a large scale. Small studies, such as a recent report that identified two cfDNA *ALK* fusions in a cohort of 102 patients show that the detection of these alterations is feasible, albeit more technically challenging than somatic mutation detection, while a longitudinal evaluation of 22 patients was able to detect *ALK* fusions in 86% at progression (18, 23, 25–27). Additionally, although numerous tumor-tissue–based studies have demonstrated mechanisms of resistance to ALKi, the use of cfDNA for the evaluation of drug resistance mechanisms in *ALK*-positive NSCLC has not been published.

In this study, we performed a survey of a laboratory cohort of *ALK*-positive patients whose cfDNA was assessed using the Guardant360 (G360; Guardant Health) assay to determine the clinical utility of plasma-based comprehensive genomic profiling for the detection of *ALK* fusions at diagnosis and for the evaluation of resistance mechanisms following disease progression on ALKi.

Patients and Methods

Sample identification

The G360 deidentified database of submitted cases with a reported diagnosis of NSCLC was queried to identify consecutive patients whose cfDNA contained *ALK* fusions or *ALK* kinase domain mutations reported out between February 2015 and November 2016. Information provided with sample submission was completed by the ordering provider and included age, sex, any accompanying tissue data, prior therapy data, and clinical status, when available. This information was abstracted to classify the samples into one of four cohorts. This research is approved by Quorum Institutional Review Board (IRB) for the generation of deidentified data sets for research purposes. For select patients, additional detail of treatments, outcomes, and tissue biopsy results were obtained from the treating physician as per local IRB guidelines.

cfDNA isolation and sequencing

cfDNA for the G360 panel was isolated as previously described at Guardant Health (Redwood City, CA; refs. 20, 21). The G360 panel is a CLIA-certified, College of American Pathologists (CAP)-accredited, New York State Department of Health (NYSDOH)-approved test that detects point mutations in up to 70 genes as well as copy number amplifications (CNA) in 18 genes, fusions in 6 genes, and small insertions or deletions (indels) in 3 genes (Table 1).

Following isolation of cfDNA, 5 to 30 ng of DNA was subjected to oligonucleotide barcoding for preparation of a digital sequencing library. This library was amplified and then enriched for the target genes using biotinylated custom baits. Each of the 70 cancer-related genes was then paired-end sequenced on an Illumina HiSeq 2500. This sequencing covered 146,000 base pairs, and each base was sequenced at average coverage depth of 10,000×. After sequencing, algorithmic reconstruction of the digitized sequencing signals was used to reconstruct the cfDNA fragments. Analytic and clinical validation has been previously reported (20, 28). The molecular barcoding and alignment allow sensitive detection of cfDNA fusion events, detected by merging overlapping paired-end reads and forming a sequenced cfDNA molecule representation; this is followed by alignment and mapping to the original sequence (28). Specific reporting thresholds were determined by retrospective and training set analyses.

The Illumina sequencing reads were mapped to the hg19/GRCh37 human reference sequence, and genomic alterations in

Table 1. Guardant360 70-gene panel

Point mutations (SNVs) with complete or critical exon coverage in 70 genes[a]									
AKT1	*ALK*	***APC***	***AR***	*ARAF*	***ARID1A***	*ATM*	***BRAF***	***BRCA1***	***BRCA2***
CCND1	***CCND2***	***CCNE1***	*CDH1*	***CDK4***	***CDK6***	***CDKN2A***	***CDKN2B***	*CTNNB1*	***EGFR***
ERBB2	*ESR1*	*EZH2*	*FBXW7*	***FGFR1***	***FGFR2***	*FGFR3*	*GATA3*	*GNA11*	*GNAQ*
GNAS	*HNF1A*	***HRAS***	*IDH1*	*IDH2*	*JAK2*	*JAK3*	***KIT***	***KRAS***	*MAP2K1*
MAP2K2	***MET***	*MLH1*	*MPL*	***MYC***	***NF1***	*NFE2L2*	*NOTCH1*	*NPM1*	***NRAS***
NTRK1	***PDGFRA***	***PIK3CA***	***PTEN***	*PTPN11*	***RAF1***	***RB1***	*RET*	*RHEB*	*RHOA*
RIT1	*ROS1*	*SMAD4*	*SMO*	*SRC*	*STK11*	*TERT*	***TP53***	*TSC1*	*VHL*

Amplifications (CNAs; 18 genes)						Fusions (6 genes)		Indels (3 genes)
AR	*BRAF*	*CCND1*	*CCND2*	*CCNE1*	*CDK4*	*ALK*	*FGFR2*	*EGFR* exon 19/20
CDK6	*EGFR*	*ERBB2*	*FGFR1*	*FGFR2*	*KIT*	*FGFR3*	*RET*	*ERBB2*
KRAS	*MET*	*MYC*	*PDGFRA*	*PIK3CA*	*RAF1*	*ROS1*	*NTRK1*	*MET* exon 14 skipping

NOTE: G360 is a CLIA-laboratory cfDNA test that detects point mutations in 70 genes and select amplifications (18 genes), fusions (6 genes), and small indels (3 genes).

[a]Boldface indicates complete exon coverage.

cfDNA were identified from the sequencing data by Guardant Health's proprietary bioinformatics algorithms. The algorithms quantify the absolute number of unique DNA fragments at a given nucleotide position, thus enabling cfDNA to be measured as a quantitative percentage of the total cfDNA (which is primarily germline cfDNA with a small amount of tumor cfDNA). The mutant allele frequency (MAF) for a given somatic mutation was calculated as the fraction of cfDNA molecules harboring that mutation divided by the total number of unique cfDNA molecules mapping to the position of the mutation and was reported as %cfDNA. The reportable range for single-nucleotide variants (SNV), indels, fusions, and CNAs in cfDNA by the G360 assay is $\geq$0.04%, $\geq$0.02%, $\geq$0.04%, and $\geq$2.12 copies, respectively (28). Plasma copy number of 2.4 is the 50th percentile in the Guardant Health database and reported as 2+, and >4.0 copies is the 90th percentile and reported as 3+.

Somatic mutation testing in tumor tissue

Tissue evaluation for *ALK* fusions and *EGFR* mutations was performed at the discretion of the patient's physician during standard of care disease management. Information regarding the specific methods of tissue testing was abstracted from the records submitted with the G360 clinical order and was not available for all patients.

RNA-based tissue NGS

Anchored Multiplex PCR-based enrichment and library preparation, examining RNA from selected regions of targeted genes in patient C4-3, was carried out using the FusionPlex Solid Tumor sequencing panel (ArcherDx, Inc.) at the University of Colorado Molecular Correlates Laboratory (CMOCO). Bioinformatics analysis was carried out using version-controlled Archer Analysis (4.1.1.7).

ALK IHC evaluation

IHC evaluation for ALK protein expression in patient C4-3 was performed at CMOCO using ALK D5F3 antibody (Ventana Medical Systems), according to the manufacturer's instructions.

Results

During the study period, samples were received from 8,744 unique patients with a diagnosis of NSCLC with 7,852 patients having cfDNA detected (89.8%). A total of 91 consecutive patients were identified who met the inclusion criteria (1.2%). Eighty-eight patients and 96 cfDNA-detected *ALK* fusions were identified across 60 institutions, both within the United States and internationally. An additional 3 patients (3 samples) were identified with an *ALK* resistance mutation but no reported *ALK* fusion in the cfDNA. Based on data provided upon sample submission, patients were separated into four cohorts (Fig. 1). Cohort 1 contained 42 patients with new discovery of an *ALK* fusion (new diagnosis or prior diagnosis with new *ALK* fusion finding). Per clinical data provided at G360 order, patients in this cohort were newly diagnosed ($N = 23$), or had been treated with a nontargeted agent following *ALK* tissue results that were negative/quantity not sufficient ($N = 7$), or had not been exposed to ALKi therapy, either by clinician report ($N = 4$), or by absence of known ALKi resistance mechanisms of G360 ($N = 8$). Cohort 2 consisted of 31 patients (and 34 samples) with known or presumed *ALK*-positive NSCLC disease progression. Patients in this cohort either had clinical information provided and known prior ALKi therapy ($N = 18$) or had a co-occurring resistance alteration, known to develop after treatment with ALKi therapy ($N = 13$). Cohort 3 included 13 patients whose samples were submitted without additional clinical information and therefore were unable to be distributed into other cohorts, and cohort 4 consisted of 6 patients with a prior *EGFR* mutation–positive lung cancer, treated with anti-*EGFR*–targeted therapy, who were found to have an *ALK* fusion by G360. Patient clinical characteristics are shown in Table 2. Each cohort and the entire patient group had an equal distribution of male/female patients. In the entire patient group, the mean age at cfDNA collection was 54 years (range, 27–84). In the 96 cfDNA *ALK* fusions, the majority were to *EML4* (85.4%), then *STRN* (6.3%), followed by *KCNQ*, *KLC1*, *KIF5B*, *PPM1B*, and *TGF* (totaling 8.3% across the five). *ALK* fusions were not identified by cfDNA in 3 patients with an *ALK* resistance mutation identified in cfDNA (3 of 91; 3.3%). All 3 patients (3 samples) were in cohort 2 (previously treated with an ALKi). In two additional cohort 2 patients with longitudinal samples (C2-1 and C2-2), the *ALK* fusion was identified in one of their serial samples, but not the other.

Newly diagnosed *ALK* fusion–positive NSCLC or new detection of *ALK* fusion not previously known (cohort 1)

The genomic landscape of cohort 1 is illustrated in Fig. 2. The *ALK* mutation status in tumor tissue was known for 10 of the 42 patients with 5 patients *ALK* positive in tissue by fluorescence *in situ* hybridization (FISH) and 5 patients *ALK* negative in tissue (4 by FISH and 1 by NGS). In 11 patients (26%) the tissue sample was insufficient to test for *ALK* status; thus, the *ALK* fusion was only identified by cfDNA. In the remaining 21 patients, *ALK* status in tissue was not provided.

Within cohort 1, 31 patients were newly diagnosed *ALK*-positive NSCLC (23 treatment-naïve, 8 treatment status unknown) and 11 patients had prior treatment for NSCLC but the *ALK* fusion was not previously identified (4 were tissue insufficient, 3 were tissue negative, 4 tissue status not reported). Among this subgroup of patients of previously unidentified *ALK* fusion ($N = 11$), the *ALK* fusion was identified in cfDNA at a median of 13.5 months (range, 5–34 months) post-initial diagnosis; 9 patients received chemotherapy prior to identification of the *ALK* fusion, 1 patient received immunotherapy and 1 received chemotherapy and immunotherapy. Clinical follow-up was available for all 3 patients with prior negative tissue testing and prior therapy (C1-2, C1-3, and C1-4, Fig. 2). Patient C1-2 had pretreatment tissue NGS of a lung lesion obtained by CT-guided core biopsy which revealed a *TP53* variant, but no other actionable mutations. Tissue was insufficient for *ALK* or *ROS1* testing by FISH. The patient was initiated on chemotherapy and while on treatment had a repeat biopsy of the liver for additional molecular testing and FISH results were negative for *ALK* and *ROS1* fusions. After the patient progressed, blood was procured for G360 testing. Results were positive for an *EML4–ALK* fusion at an MAF of 0.9%. Based on these results, the patient was started on crizotinib. Pre-crizotinib CT scan of a representative lesion and repeat imaging performed at 10 weeks demonstrated a dramatic response (Supplementary Fig. S1A and S1B). The patient remained on crizotinib for 7 months before progression.

Patient C1-3 had pretreatment tissue testing by rtPCR and FISH, which was negative for *EGFR* mutations and *ALK* fusions. Over a 3-year period, the patient was treated with

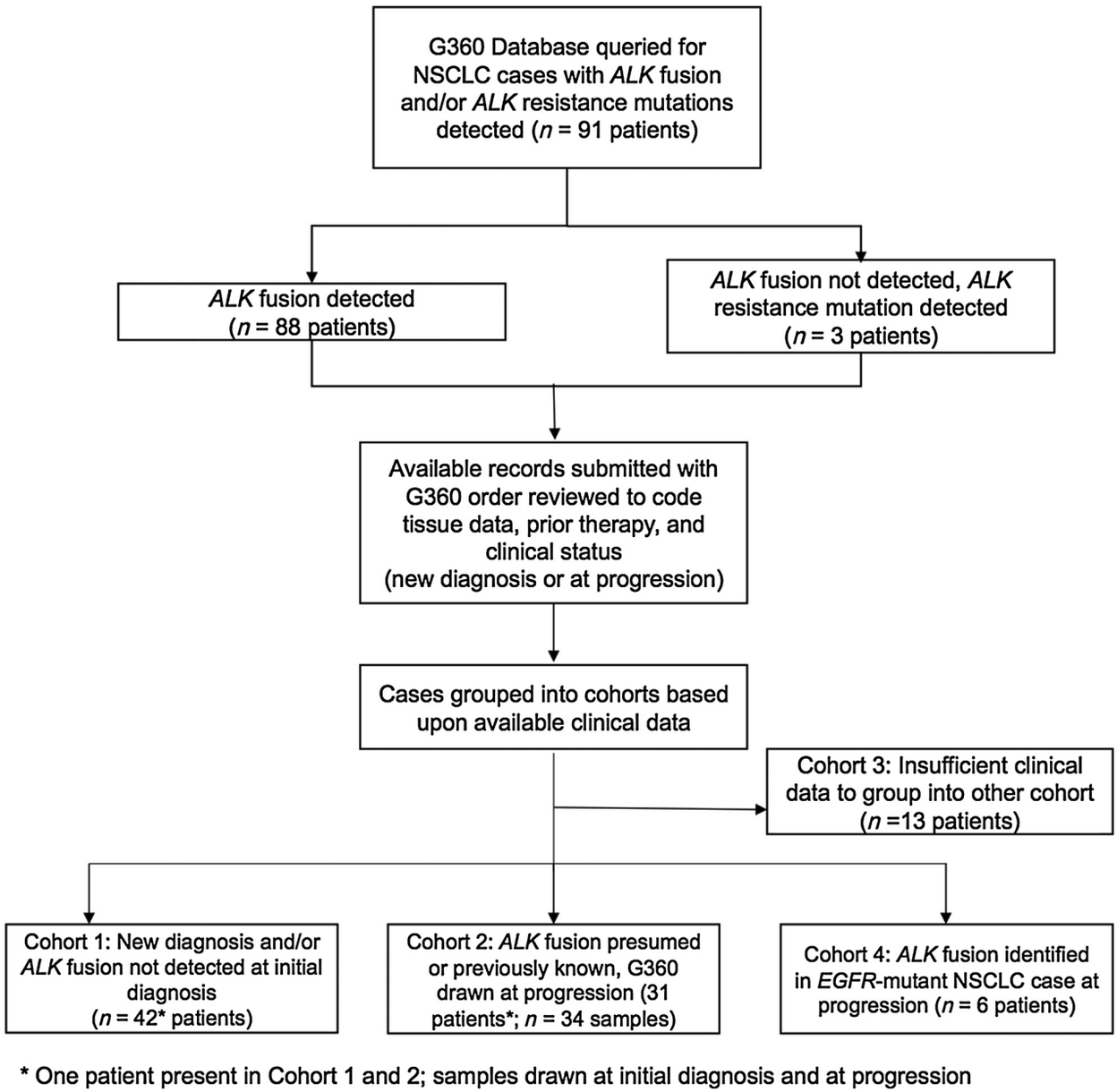

Figure 1.
CONSORT diagram.

chemotherapy and immunotherapy. At progression, G360 identified an *EML4–ALK* fusion at 0.3%. After progression on second-line chemotherapy, treatment was switched to crizotinib with a response to therapy that was still ongoing at the most recent imaging (Supplementary Fig. S1C and S1D).

Patient C1-4 had local laboratory pretreatment FISH testing of a bone metastasis that was deemed of insufficient quantity. Follow-up targeted NGS testing of a lymph node was negative for any *ALK* fusions or other oncogenic mutations. The patient was treated with stereotactic radiation to the brain followed by palliative chemotherapy until progression. Surgery was performed for spinal cord decompression and was followed by palliative radiation to the spine and additional sites of bony metastases, followed by immunotherapy with pembrolizumab until progression. Tissue NGS was again attempted locally and was positive for an *ALK* fusion. G360 ordered at the same time was also positive for the *EML4–ALK* fusion at 0.1%. The patient was started on crizotinib until progression at 6 months, at which time he was transitioned to alectinib and continues to have stable disease after 8 months of treatment.

Other genomic alterations, including SNVs and CNAs, were also identified in cohort 1 samples. *TP53* alterations were identified in 18 of 42 patients (43%) in cohort 1. This is consistent with prior reports of the frequency of *TP53* alterations in lung

Table 2. Patient demographics

	Cohort 1 (new Dx or new *ALK* Dx)	Cohort 2 (ALK TKI progression)	Cohort 3 (unknown clinical status)	Cohort 4 (EGFR TKI progression)	All[a]
Patients (*N*)	42	31	13	6	91
Gender					
Female	22 (52%)	15 (48%)	6 (46%)	3 (50%)	46 (51%)
Male	20 (48%)	16 (52%)	7 (54%)	3 (50%)	45 (49%)
Age, years					
Average (range)	54.6 (27–84)	50.7 (27–73)	58.6 (46–82)	61.2 (43–71)	54 (27–84)
cfDNA-detected fusion (by sample)					
EML4–ALK	40	24	13	5[b]	82
STRN–ALK	—	—	—	6[b]	6
KCNQ–ALK	—	1	—	—	1
KLC1–ALK	—	3	1	—	4
KIF5B–ALK	1	—	—	—	1
PPM1B–ALK	—	1	—	—	1
TFG–ALK	1	—	—	—	1
Not detected	—	5	—	—	5
Total	42	34	14	11	101

[a]One patient in cohorts 1 and 2 counted once in "All".
[b]One patient in cohort 3 had *EML4 and STRN–ALK* fusions.

cancer and similar to that seen in *ALK* fusion–positive NSCLC (10, 29, 30). One patient had co-occurring *KRAS* mutations (G13D and V14I) which were observed in trans with each other. Three patients had CNAs in one or multiple genes. Patient C1-17 demonstrated an *ERBB2* CNA, patient C1-27 demonstrated a *BRAF* and *PIK3CA* CNA, and patient C1-16 demonstrated CNA in *BRAF*, *CCND1*, *CDK6*, *EGFR*, *KIT*, *MET*, and *PDGFRA* (Fig. 2). As *PDGFRA*/*KIT* are located on chromosome 4 and *BRAF*/*EGFR*/*MET*/*CDK6* are on chromosome 7, this may reflect aneuploidy in the tumor cell as opposed to focal gene CNA.

Known or presumed *ALK* fusion–positive patients whose cfDNA had been drawn at progression (cohort 2)

Cohort 2 contained 31 patients with a known or presumed *ALK* fusion who had received an ALKi (Fig. 3). Overall line of treatment and complete treatment history is unknown. Resistance mutations in the *ALK* kinase domain were detected in 16 patients (52%). The most common resistance mutations identified were G1202R (8 patients), F1174C/V/L (6 patients), and I1171T/N (5 patients).

In the 8 patients with a G1202R mutation, the most recent treatment was alectinib for one; ceritinib, then alectinib in a second patient; chemotherapy (unspecified) for a third; and not provided for the remainder. The MAF (0.27% and 0.14% for patients C2-1_2 and C2-3_2, respectively) is comparatively low for both patients for the G1202R mutations compared with other co-occurring mutations, consistent with more recent development (Fig. 3).

For the *ALK* mutation F1174C/V/L, the most recent *ALK* tyrosine kinase inhibitor (TKI) was known for 4 of the 6 and included ceritinib then alectinib in 2 patients, crizotinib in 1 patient, and lorlatinib in 1 patient. In the 5 patients in which I1171T/N was found, the most recent treatment was known in 2 patients, 1 patient received chemotherapy and in the second patient, two separate I1171 mutations were found at different treatment time points: (i) after treatment with crizotinib, I1171T was identified (MAF 4.71%) and (ii) after treatment with ceritinib then alectinib, I1171N was identified (MAF 0.29%). The I1171T was no longer identified at the second analysis.

Using prior treatment as a comparator, in the 9 patients who received crizotinib, 2 (22%) developed resistance mutations, 1 with a single mutation (F1174V, C2-3_1) and 1 patient with a dual mutation (G1269A and I1171T, C2-1_1). Seven patients received alectinib as their most recent treatment, and 4 (54%) demonstrated resistance mutations; 2 patients with single mutations (G1202R, C2-3_2; L1196Q, C2-7), and 2 patients with 3 mutations each (I1171N, F1174L, and G1202R in C2-1_2; F1174L, C1156Y, and D1203N in C2-2_2).

Three patients had two separate postprogression cfDNA evaluations after progression on different treatments (C2-1, C2-2, and C2-3). Interestingly, the second assessments demonstrated an entirely different complement of resistance mutations for all 3 patients (Fig. 3). The MAFs for these samples is shown demonstrating the relative frequencies of each kinase domain mutation.

In 6 patients, concurrent resistance mutations were identified; 4 patient samples demonstrated 3 mutations (C2-1_2, C2-2_2, C2-20, C2-24) and 3 patient samples demonstrated 2 concurrent mutations (C2-2_1, C2-28, C2-29). Two of the 3 patients described in the prior paragraph who had serial testing developed a different spectrum of resistance mutations in the later sample. In 5 samples, an *ALK* kinase domain mutation was identified in cfDNA but the *ALK* fusion was not detected in cfDNA despite prior tissue testing showing an *ALK* fusion (samples denoted by a "T" in Fig. 3).

In addition to mutations in the *ALK* kinase domain, multiple additional cancer-related genes demonstrated mutations or CNAs. Seven patients had a mutation in a potential alternative oncogenic driver in addition to detected *ALK* fusion. Specifically, 4 patients had mutations in the RAS pathway, including 2 with *KRAS* G12C/V (C2-8, C2-24), 1 patient with *HRAS* Q61L (C2-12) and 1 patient with *KRAS* G13C (C2-31). Three patients had individual mutations in *BRAF* V600E (C2-23), *EGFR* E330K (C2-15), or a *MET* splice-site mutation (C2-14). Two patients with *ALK* kinase domain mutations also demonstrated an activating mutation in an alternate oncogene [*BRAF* V600E (C2-23) and *KRAS* G12C/V (C2-24)] and 5 were found to have CNAs (C2-1_1/2, C2-2_2, C2-3_2, C2-25, C2-28).

Across the cohort, 8 patients demonstrated CNAs. These were primarily single gene amplifications with C2-2_2 demonstrating amplification of *CCND2* and *FGFR2* and patient C2-31

	ALK Tissue Status	EML4-ALK fusion	KIF5B-ALK fusion	TFG-ALK fusion	APC SNV	AR SNV	ARID1A SNV	ATM SNV	CDKN2A SNV	CTNNB1 SNV	KRAS SNV	NF1 SNV	NFE2L2 SNV	PIK3CA SNV	SMAD4 SNV	TP53 SNV	ERBB2 AMP	BRAF AMP	CCND1 AMP	CDK6 AMP	EGFR AMP	KIT AMP	MET AMP	PDGFRA AMP	PIK3CA AMP	Prior Systemic Therapy?	Days between dx and G360
C1-1	**NEG**	0.3														0.79										**None**	**52**
C1-2	**NEG**	0.94					0.43									1.48										**Chemotherapy**	**133**
C1-3	**NEG**	0.28														0.16										**Chemo /Immunotherapy**	**1056**
C1-4	**NEG**	0.05																								**Immunotherapy**	**285**
C1-5	**NEG**	0.33																								**Unknown**	**78**
C1-6	POS	0.56																								None	7
C1-7	POS	0.12																								None	13
C1-8	POS	0.07																								Unknown	6
C1-9	POS	0.17											0.78			0.76										Unknown	21
C1-10	POS	0.58*								0.63																Unknown	207
C1-11	QNS	1.19																								None	2
C1-12	QNS	3.32			0.39											0.42										None	5
C1-13	QNS	0.19							0.33							0.38										None	6
C1-14	QNS	0.26														1.29										None	19
C1-15	QNS	0.49*									0.16#					0.34										None	27
C1-16	QNS	10.5																2.93	2.46	2.6	2.62	2.56	2.36	2.46		None	Unk
C1-17	QNS	0.04															2.19									Chemotherapy	185
C1-18	QNS	1.92														0.44										Chemotherapy	257
C1-19	QNS	0.06*																								Chemotherapy	614
C1-20	QNS		0.14												0.2	1.25										Chemotherapy	771
C1-21	QNS	0.08																								Unknown	43
C1-22	Unk	0.05																								None	1
C1-23	Unk	0.04						0.11																		None	2
C1-24	Unk	0.34																								None	2
C1-25	Unk	0.82														1.59										None	3
C1-26	Unk	0.67														0.57										None	5
C1-27	Unk	2.52																2.23						2.21		None	6
C1-28	Unk			0.05																						None	8
C1-29	Unk	0.84														0.13#										None	10
C1-30	Unk	0.14														1.86										None	11
C1-31	Unk	0.26														0.83										None	12
C1-32	Unk	1.94										0.13														None	14
C1-33	Unk	3.01																								None	14
C1-34	Unk	0.14														1.56										None	21
C1-35	Unk	12.5																								None	28
C1-36	Unk	0.32																								Chemotherapy	22
C1-37	Unk	0.05				0.78	0.61								0.49											Chemotherapy	27
C1-38	Unk	0.06																								Chemotherapy	53
C1-39	Unk	0.13														0.12										Chemotherapy	560
C1-40	Unk	0.16														0.27										Unknown	11
C1-41	Unk	0.2																								Unknown	77
C1-42	Unk	0.13																								Unknown	115
	Total:	40	1	1	1	1	2	1	1	1	1	1	1	1	1	18	1	2	1	1	1	1	1	1	1		

Figure 2.

Cohort 1 (newly identified *ALK* fusion) genomic landscape. Individual patient results and cfDNA identified alterations for cohort 1, newly identified *ALK* fusions. Tumor tissue *ALK* status was known for 21 (50%) of cohort 1 cases: 5 negative (NEG), 5 positive cases (POS), 11 insufficient tissue to perform analysis or unable to obtain tissue for analysis (QNS). The remainder of the samples had tissue status that was unknown (Unk). Alterations identified are in the following columns and denoted by color: blue shades, fusion; green, *RAS/RAF/EGFR/MET* variant; red, amplification; light gray, tumor suppressor/other pathway gene mutations. Asterisk (*) indicates instances in which only the reciprocal *ALK–EML4* fusion was detected in cfDNA. If known, the prior systemic therapy is listed, as are the days between diagnosis and blood draw for Guardant360 (median 21 days; range, 1–1,056 days).

demonstrating amplification of *EGFR*, *MYC*, and *FGFR1*. Notably, *ALK* was shown to regulate the *MYC* signaling axis and together with these results suggest that *MYC* amplification may be able to partially bypass ALK signaling (31). Patient C2-8 demonstrated amplification in 7 genes. Similar to patient C1-16, amplified genes were clustered on the same chromosome (*BRAF*/*EGFR*/*MET*/*CDK6* are located on chromosome 7 while *CCND2*/*KRAS*/*CDK4* are located on chromosome 12); therefore, this likely represented aneuploidy as opposed to independent focal gene amplification events.

As noted above, 3 patients underwent more than one cfDNA evaluation during their disease trajectory. The shifting resistance mutation profile of 1 of these patients, C2-3, is illustrated in Fig. 4. At diagnosis, the patient's cfDNA and tissue demonstrated an *EML4–ALK* fusion and was noted to also have a mutation in *ARID1A*. The patient began treatment with crizotinib, but switched to ceritinib due to side effects. After progression on ceritinib the patient's cfDNA was again evaluated. In addition to the original fusion gene, the *ALK* F1174C resistance mutation was also detected. The patient was then started on alectinib to which the patient clinically responded. At progression, cfDNA was reassessed; the original fusion gene was again identified; however, the F1174C mutation was no longer identified in cfDNA, but G1202R was present.

Recently, Lin and colleagues and Ou and colleagues separately presented data demonstrating a difference in the development of *ALK* kinase domain resistance mutations depending on the specific *EML4–ALK* fusion variant (32, 33). We evaluated the data based on the two most common variants, *EML4* exon 13 to *ALK* exon 20 (variant 1, $n = 9$) an *EML4* exon 6 to *ALK* exon 20 (variant 3, $n = 7$). In cohort 2, ALK kinase domain mutations were observed in 4/9 (44.4%) of samples with

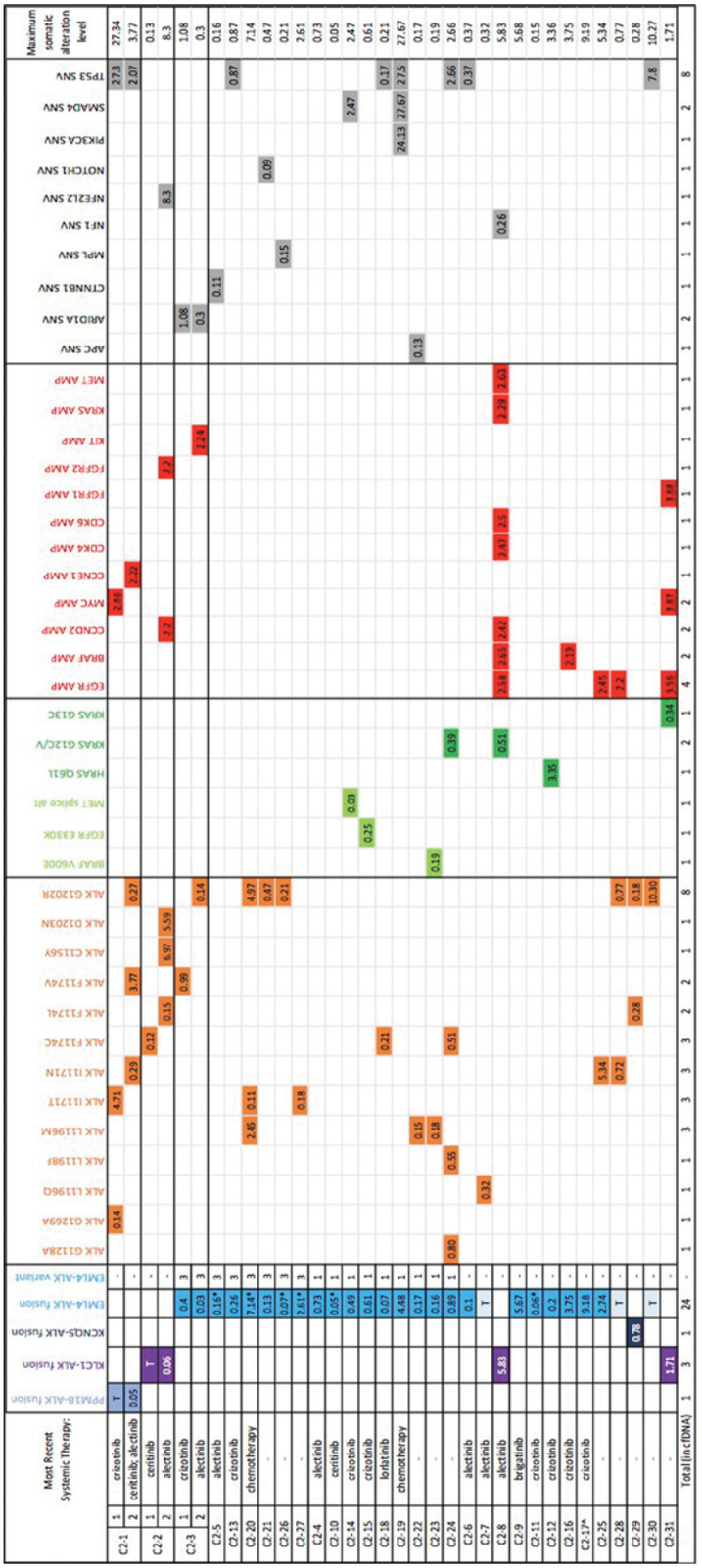

Figure 3.

Cohort 2 genomic landscape. Individual patient results are shown in rows, and cfDNA identified alterations are shown in each column. Patients C2-1, C2-2, and C2-3 had multiple progression samples collected, denoted as 1 and 2. The most recent treatment is noted in the following column, if known. The mutational allele frequency (MAF) is shown in each box for fusions, resistance mutations, and somatic mutations in alternative oncogenes. The maximum somatic alteration allele frequency for each sample is shown in the far right column. "T" denotes fusion previously detected in tissue but not detected in cfDNA at progression. Asterisk (*) indicates instances in which the reciprocal *ALK–EML4* fusion was also detected in cfDNA. Color legend: blue/purple shades, fusion; orange, on-target resistance mutation; green, *RAS/RAF/EGFR/MET* mutation; red, amplification. Sample C2-17 is the progression sample from patient C1-26 in cohort 1.

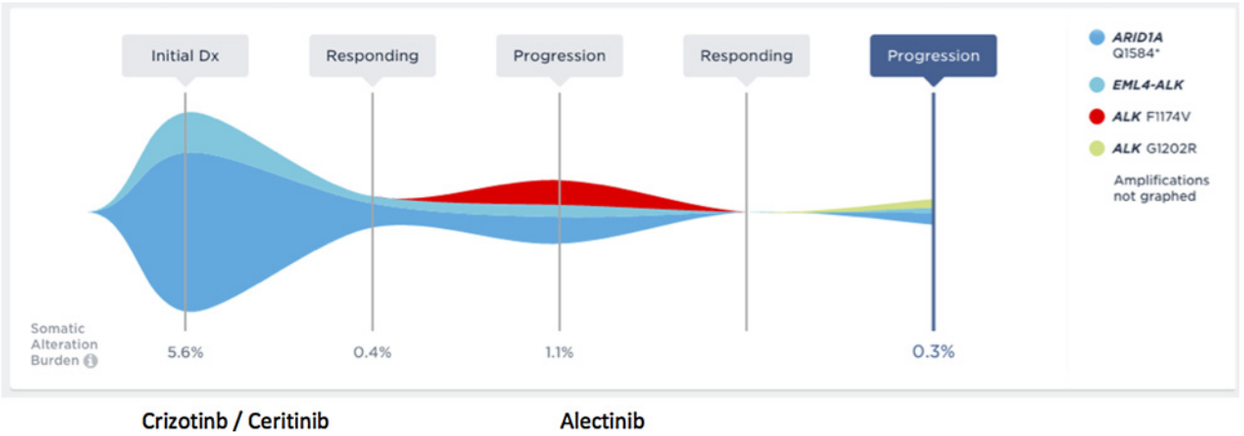

Figure 4.
NSCLC case with multiple cfDNA time points across disease trajectory. Patient C2-3: At initial diagnosis, *EML4–ALK* fusion was detected in cfDNA (and tissue). Crizotinib was initiated with 32% reduction in target lesion in 3 months. Treatment was switched to ceritinib due to side effects. G360 was drawn again when progression occurred on ceritinib, and the original fusion was detected along with *ALK* F1174V, a mutation conferring resistance to crizotinib and ceritinib. Alectinib was then initiated, and after initial response, progression was noted and G360 was drawn again: F1174V was no longer present in circulation, but G1202R was identified, a mutation conferring resistance to all FDA-approved ALKi but predicted to be sensitive to lorlatinib and brigatinib. Lorlatinib currently is available only by clinical trial.

variant 1 and 6/8 (75%) of samples with variant 3 (P = 0.2145). G1202 was observed in 0/9 (0%) variant 1 and 4/8 (50%) variant 3 samples. (P = 0.0186). Cohort 2 breakpoint data for each sample is listed in Supplementary Table S1.

Patients with unknown clinical status (cohort 3)

For 14 patients, we did not have sufficient clinical data to classify them into one of the other cohorts. Their molecular characteristics are illustrated in Fig. 5. Thirteen (93%) patients had fusions to *EML4* and 1 patient had a *KLC1–ALK* fusion. Eight patients demonstrated SNVs in other gene. No CNAs or resistance mutations were identified.

Newly acquired ALK fusions as resistance mechanisms (cohort 4)

Cohort 4 contained 6 patients who were known to have an *EGFR*-activating mutation by tissue testing (exon 19 deletion in 4 patients and L858R in 2 patients; Fig. 6). These patients demonstrated progression on *EGFR* TKI after a median of 2.5 years (range, 0.5–7.4 years). Notably, cfDNA at progression on an

Alteration Type	ALK Tissue Status	EML4-ALK fusion	KIF5B-ALK fusion	TFG-ALK fusion	KLC1-ALK fusion	APC SNV	AR SNV	ARID1A SNV	ATM SNV	CDKN2A SNV	CTNNB1 SNV	KRAS G13D	KRAS V14I	MET SNV	MPL SNV	NF1 SNV	NFE2L2 SNV	PTEN SNV	PIK3CA SNV	SMAD4 SNV	TP53 SNV	ERBB2 AMP	BRAF AMP	CCND1 AMP	CDK6 AMP	EGFR AMP	KIT AMP	MET AMP	PDGFRA AMP	PIK3CA AMP
# of Cohort 3 pts	14	13	0	0	1	0	0	0	1	1	0	0	0	1	1	0	0	1	0	0	4	0	0	0	0	0	0	0	0	0
Cohort 3 Pt: C3-1	Unk	0.48							1.51																					
C3-2	Unk	2.96								9.78								0.29												
C3-3	POS	0.15												0.23																
C3-4	Unk	0.60*													0.15						0.83									
C3-5	Unk	0.23													1.22															
C3-6	Unk	0.16																			1.22									
C3-7	POS	4.85																			27.6									
C3-8	Unk	4.74																			0.11									
C3-9	Unk	0.3																												
C3-10	Unk	2.86																												
C3-11	Unk	0.21																												
C3-12	Unk	0.15																												
C3-13	Unk	0.23																												
C3-14	Unk				0.4																									

Figure 5.
Genomic landscape of cohort 3. Cohort 3, genomic landscape for patients with unknown clinical status. Individual patient results are shown in rows, and cfDNA-identified alterations are shown in each column. The second column lists the tissue status; POS, FISH positive; NEG, FISH negative; UNK, unknown. Color legend: blue shades, fusion; green, *RAS/RAF/EGFR/MET* mutation; red, amplification; light gray, tumor suppressor/other pathway gene mutations. Asterisk (*) indicates instances in which only the reciprocal *ALK–EML4* fusion was detected in cfDNA.

A

Alteration type	EGFR E19d/L858R	EGFR T790M	EML4-ALK fusion	STRN-ALK fusion	NRAS G13V	EGFR AMP	MYC AMP	CDK6 AMP	MET AMP	BRAF AMP	CCNE1 AMP	KRAS AMP	PDGFRA AMP	PIK3CA AMP	AKT1 SNV	CDKN2A SNV	PIK3CA SNV	TP53 SNV	Maximum somatic alteration level	Most recent treatment (or full treatment history if more than one listed)
C4-1 (exon 19 deletion)	18.65	0.49	0.13			2.62		3.01		2.55			2.45	2.46				46.13	46.13	not provided
*C4-2_1 (L858R)	21.86	3.67	0.78	0.25		2.53												5.17	21.86	osimertinib
*C4-2_2	62.48	8.47	2.39			5.54	2.47	2.6	4									29.77	62.48	not provided
*C4-2_3	50.56	9.85	3.38			4.21												16.54	50.56	not provided
*C4-2_4	77.3	15.52	8.34	0.06		9.31	2.93		3									63.17	77.30	not provided
*C4-3 (exon 19 deletion)	15.34	0.54		0.14		2.49	23.6		6							11.9		26.59	26.59	erlotinib
C4-4 (L858R)	1.35	0.42		0.1	0.2													0.95	1.35	osimertinib
*C4-5 (exon 19 deletion)	9.8	T		3.02		2.57	2.84				2.28				10.2		2.33	9.39	10.19	afatinib, osimertinib, nivolumab
C4-6 (exon 19 deletion)	6.15			3.67			2.44					2.23						17.84	17.84	pemetrexed/carboplatin
Total tests	9	8	5	6	0.2	7	5	2	3	1	1	1	1	1	1	1	1	9		

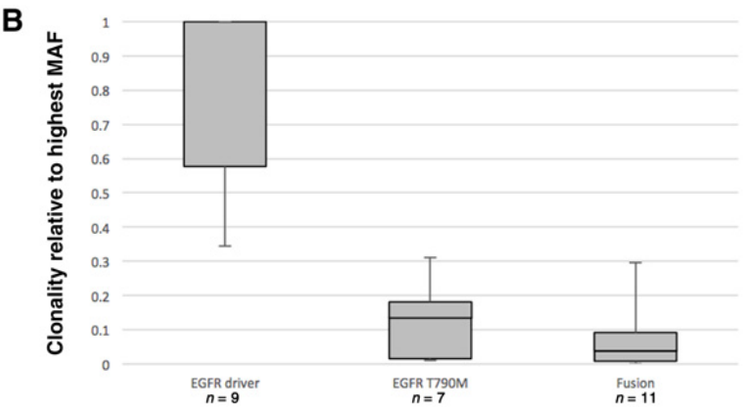

Figure 6.

Cohort 4 genomic landscape. **A,** Individual patient results are shown in rows, and cfDNA identified alterations are shown in each column. The *EGFR* mutation subtype is shown in parentheses. The most recent treatment is noted in the far-right column. The mutant allele frequency (MAF) is shown in each box for fusions, resistance mutations, and somatic mutations in alternative oncogenes. Color legend: blue shades, fusion; orange, on-target resistance mutation; green, *RAS/RAF/EGFR/MET* mutation; red, amplification; light gray, tumor suppressor/other pathway gene mutations Eleven *ALK* fusions (6 *STRN–ALK*, 5 *EML4–ALK*) were identified in 6 patients, drawn when progression occurred on an *EGFR* TKI (of 1450 NSCLC cases with *EGFR* driver mutations in the Guardant database). *EGFR* T790M was also present in 5 of 6 patients (4 by cfDNA in 7 tests, 1 by tissue [T]) along with multiple amplification events. In 3 patients (*), tissue testing results from initial diagnosis were available and were *EGFR* positive, *ALK* fusion negative. In patient C4-3, progression tissue biopsy, the presence of the *STRN–ALK* fusion was confirmed by an RNA-based NGS assay. **B,** Relative to the highest MAF variant in circulation, the *EGFR* driver mutations appear to be clonal, whereas both T790M and the fusions appear to be subclonal. This information, combined with available treatment-naïve tissue testing results, suggests that the *ALK* fusion events are emergent alterations.

EGFR TKI demonstrated *ALK* fusions: 1 patient had both an *EML4–ALK* and *STRN–ALK* fusion, 1 patient had an *EML4–ALK* fusion, and 4 patients had *STRN–ALK* fusions. The initial diagnostic biopsy tissue testing results were available for 3 patients and were positive for the same *EGFR*-activating mutation identified on cfDNA, *EGFR* T790M negative, and *ALK* negative by FISH in all three (C4-3, C4-4, and C4-5). Five patients also demonstrated an *EGFR* T790M resistance mutation (four by cfDNA and one by tissue). The most recent treatment at the time of the cfDNA draw included osimertinib for 2 patients, erlotinib for 1 patient, chemotherapy for 1 patient, nivolumab (previous afatinib and osimertinib) for 1, and unknown for the remaining patient. As shown in Fig. 6, cfDNA evaluation also identified 2 to 5 gene amplification events in 5 patients and SNVs in *AKT1* (1), CDKN2A (1), PIK3CA (1), and TP53 (6).

MAF of variants in circulation was also evaluated for patients in cohort 4. Figure 6B displays the clonality relative to the highest MAF in the sample. In this analysis, the *EGFR*-activating mutation had the highest relative MAF, the *EGFR* resistance mutation, T790M, had a lower relative MAF, consistent with a subclonal population and the ALK fusion protein had even lower clonality, consistent with the presence of a small subclonal population. The available "*ALK*-negative" tissue results from initial diagnosis combined with the low subclonality of the cfDNA-detected *ALK* fusion at progression suggest that the fusion event is either a resistance mechanism emerging under treatment selection or represents a small subclone not detected at initial diagnosis that was selected for under *EGFR*-targeted therapy.

Patient C4-3 in this cohort is a 43-year-old Caucasian man who was initially diagnosed with advanced NSCLC after presenting with back pain. NGS revealed an *EGFR* exon 19 deletion as well as a *TP53* mutation (L114*). Testing for alterations in *ALK, ROS1, RET, MET* amplification as well as the 26-gene TruSight NGS tumor sequencing panel was negative. FISH testing on this diagnostic biopsy sample was also negative for an *ALK* rearrangement. The patient was started on erlotinib and bevacizumab. After 6 months, imaging demonstrated osseous progression. Given the challenge of molecular analysis of bone biopsies, G360 was

performed and demonstrated the original *EGFR* exon 19 deletion and *TP53* mutation as well as a T790M mutation, an *EGFR* amplification, a *CDKN2A* mutation (H83Y), *MYC* mutation (D173A), amplification (3+), *STRN–ALK* fusion, and *MET* amplification (3+). Notably, a biopsy performed after cfDNA analysis confirmed the presence of an *STRN–ALK* fusion using an RNA-based NGS assay (Supplementary Fig. S2). Further, the *EGFR* exon 19 deletion and the T790M mutation were demonstrated by DNA-based tumor NGS, the ALK expression was confirmed by IHC (Supplementary Fig. S3), and *MET* amplification was confirmed by FISH (MET/CEP7 ratio 5.04). Given the *MET* amplification and the *ALK* fusion, the patient's therapy was changed to osimertinib and crizotinib with radiation to painful sites of disease in the thoracic and lumbar spine. The treatment was tolerated well and resulted in radiologically stable disease for eight months, demonstrating the clinical benefit of plasma-based NGS testing to identify resistance mechanisms and determine next lines of treatment.

Discussion

In this retrospective study, we examined the efficacy of using cfDNA to (i) identify *ALK* fusions in plasma and (ii) identify resistance mechanisms through utilization of a targeted 70-gene cfDNA NGS test.

In cohort 1 patients (either newly diagnosed lung cancer or in whom an *ALK* fusion gene was not previously identified), we were able to demonstrate an *ALK* fusion in 16 patients who had previously been reported as tissue negative or tissue insufficient, in addition to confirming the molecular diagnosis in five patients and providing an *ALK* fusion diagnosis in 25 patients. Consistent with our finding, a recent tissue-based study reported a FISH false-negative rate of 35% in a cohort of 47 patients who were found to be *ALK* positive by NGS, suggesting that NGS-based assessment for *ALK* fusions may be warranted in patients with higher probability of *ALK* fusion and whose FISH analysis is negative (34). The importance of this is illustrated by 3 patients in our cohort who, despite a negative *ALK* FISH were *ALK* positive by cfDNA and went on to respond to ALKi treatment. Clinical data were not available for the remaining two tissue-negative but cfDNA-positive *ALK* fusion patients. Notably, among the 8 patients who received prior lines of non-ALKi therapy due to tissue insufficiency/false-negative results, two received immunotherapy which is known to have an inferior response to treatment in *ALK* positive lung cancer (35). For the 11 cases whose tissue was "quantity not sufficient" for biomarker testing, the cfDNA analysis salvaged the molecular testing by providing an oncogene result without requiring an additional biopsy. Thus, across this cohort, the use of cfDNA to complement tissue testing provided effective treatment options in these patients. Additionally, the utility of cfDNA in fusion detection is not limited to *ALK* fusions. A recent study of tumors with *RET* fusions used cfDNA to identify several patients in their cohort (36). As the current cohort of patients was selected based on a positive cfDNA *ALK* result, we do not have an accurate estimate for the false-negative rate. Therefore, this testing should be viewed as a rule-in versus a rule-out test.

We also explored the genomic landscape of known or presumed *ALK*-positive patients whose cfDNA was interrogated at the time of disease progression. In this cohort, a possible mechanism of resistance was identified in 24 of the 31 patients (77%). Recently reported cohorts of *ALK*-positive patients have identified similar percentages of resistance mechanisms (including kinase domain mutations, alternative oncogenic mutations, and copy number gains) in patients who have progressed on at least one type of ALKi therapy (10, 37, 38).

In evaluation of resistance mutations, 16 of 31 patients (51.6%) were identified with at least one mutation in the kinase domain. This result is similar to other studies reporting between 44% and 56% of patients with kinase domain mutations (10, 27, 37, 38). Notably, 7 of the 16 (43.8%) patients with resistance mutations in *ALK* demonstrated more than one mutation. This contrasts with a recently published series of *ALK*-positive patients who underwent tissue-based NGS after progression on second-generation ALKi in which 6 of 48 (12%) patient specimens contained compound mutations (10). The increased percentage in our cohort may reflect the nature of cfDNA, which contains tumor DNA shed from multiple tumor sites throughout the body, whereas tissue biopsy of a single site may not fully represent tumoral heterogeneity both within an individual lesion and across multiple metastatic sites (13–15). Therefore, it is important to consider that cfDNA may present a more accurate picture of tumor heterogeneity and the challenges of overcoming resistance to targeted therapy given multiple complementary mechanisms of resistance.

In three patients from cohort 2 we collected two cfDNA time points during the patients' disease trajectory. In each case, the second cfDNA assessment demonstrated a complete shift in the resistance mutation spectrum, with *ALK* fusions and/or initial *ALK* resistance alterations becoming undetectable and new *ALK* resistance alterations appearing. Additionally, in 2 patients, the number of resistance mutations increased (C2-1 and C2-2). The shifts in the mutation spectrum likely reflect the selective pressure of different *ALK* targeted agents. This again illustrates the benefit of using cfDNA resistance profiles to give a picture of the complexity of resistance to targeted therapy in a heterogeneous tumor cell population.

The interpretation of MAFs in cfDNA is evolving. The MAFs for cohort 2 are shown in Fig. 3. In general, the initial driver alterations, often a *TP53*-inactivating mutation and *ALK* fusion, are at the highest MAF pretreatment, suggesting an early, truncal event. At progression on crizotinib, the *ALK* resistance mutation is often at lower MAF, reflecting its more recent development as a branched event. In instances in which there are multiple *ALK* resistance mutations identified in a single sample at progression on a given ALKi, it is interesting to consider whether the resistance mutation with the highest allele frequency indicates the mutation that is driving resistance or reflects more nuanced factors that influence tumor DNA shedding, such as location, tumor size, or blood supply. Regardless, high prevalence in blood does not necessarily equate to being the dominant driver of resistance as additional somatic mutations both within the *ALK* kinase domain and in other genes may shift resistance and sensitivity profiles. Additional studies need to be done to further clarify the utility of using MAF in longitudinal cfDNA interpretation.

Finally, in samples from 5 patients from cohort 2 (14.7% of cohort 2 samples), the *ALK* fusion was not detected, but a resistance mutation was identified in the *ALK* kinase domain indicating the presence of the *ALK* fusion despite the absence of

detection by cfDNA. In 2 of these patients, the ALK fusion was detected in the cfDNA at a different time point. The sensitivity of fusion detection in cfDNA is known to be lower than that for SNVs or indels. cfDNA is highly fragmented, making it more prone to interference leaving insufficient mappable sequence to identify the fusion event [e.g., complex fusion events involving multiple partners or generation of random sequences (due to double strand break rescue gap-fill) which do not map to the human genome] and fusion molecules can be lost due to fusion hybrid capture inefficiencies. Due to these technical explanations, and known biological reasons for low detection rate (e.g., low tumor DNA shedding on treatment), as mentioned above, cfDNA should be utilized as a rule-in versus a rule-out test. In these 5 patients, the identification of the *ALK* resistance mutation is pathognomonic for the presence of the *ALK* fusion, even if the latter is present below the reportable range for the cfDNA assay.

In cohort 4, interrogation of cfDNA identified *ALK* fusions in 6 patients known to have *EGFR*-mutant NSCLC who had progressed on prior therapy. The detection of *ALK* fusions as a mechanism of resistance to EGFR TKI therapy has been previously reported (39). Conversely, *EGFR*-activating mutations have been identified as a mechanism of resistance in patients initially identified as *ALK* fusion positive, both in this series (patient C2-15; Fig. 3) and elsewhere (40). The incidence of emerging *ALK* fusions in patients treated with EGFR TKIs is unknown, but is likely infrequent. In a recently presented cohort of over 5,000 patients with advanced treatment-naïve and progressing NSCLC, tested by G360, 26.4% ($N = 1{,}361$) had detectable EGFR driver alterations. cfDNA T790M was detected in 654 patients (48%). In the current study, we reviewed the results for over 8,000 treatment-naïve and progressing patients with advanced NSCLC and identified only 6 patients with an *EGFR*-activating mutation and *ALK* fusion at progression on prior treatment (41). The MAFs demonstrate the clonality of the primary oncogenic driver (*EGFR*), and the subclonal populations of the T790M resistance mutation, and the *ALK* fusion, each with decreasing MAFs.

As illustrated in Table 2, the majority of fusion partners identified across cohort 4 patients were *EML4*; however, in cohort 4 we identified 5 *STRN* fusion events in 6 patients. The *STRN–ALK* fusion has been previously reported in thyroid cancer, with an increased frequency in poorly differentiated (9%) and anaplastic thyroid cancer (4%) compared with papillary thyroid cancer (1.6%; refs. 42, 43). *STRN–ALK* fusions have been reported in patients with NSCLC, with a recent case report describing a patient with this rare fusion event and resistance to alectinib (44). This is the first report of *STRN–ALK* fusions in a cohort of patients treated with EGFR TKIs. The true incidence of these *STRN* fusions in NSCLC as oncogenic drivers or potential therapeutic resistance alterations remains unknown, though likely very rare. The *STRN* gene is located on chromosome 2 and encodes a calmodulin binding protein thought to be involved in Ca2+ depending scaffolding (45). It was initially localized in neurons and its coiled-coil domain has been previously reported to lead to MAPK signaling via dimerization (42). Its high prevalence in this molecularly defined cohort may indicate a possible preferential fusion event in patients who develop *ALK* fusions in response to the selective pressure of an EGFR TKI. Notably, in patient C4-3's tumor sample, this fusion was confirmed by two orthogonal methods, RNA NGS and ALK IHC. The patient example from this cohort achieved prolonged disease stabilization with dual TKI therapy. Efficacy of combination treatment with ALKi and *EGFR* inhibitors has been demonstrated previously in other clinical situations but has been limited by toxicity (46–48). It is notable that several groups have now reported the finding of oncogene fusions involving *ALK*, *RET*, *NTRK1*, and *FGFR3* fusions in the setting of EGFR TKI resistance and suggests that broad testing for these targetable alterations at resistance (in addition to *EGFR* T790M) may allow for attempts to overcome resistance using combinations of targeted agents (39, 49–52).

This study has several limitations. First, this is a retrospective analysis reliant on clinical information provided on sample submission. Therefore, complete treatment history and clinical follow-up is not available (and cannot be verified) for all patients. This includes patient demographic information, type and length of prior therapies, local tissue testing modality, and prior molecular testing results both at diagnosis and progression rebiopsy. Further, there are limitations to the cfDNA platform, including the identification of multiple subclonal populations, which may not be clinically relevant to resistance. Additionally, given G360 is a clinical cfDNA assay, only *ALK* fusion events that occur with partners with known biologic significance are reported. Finally, in this study we identified 6 patients in cohort 2 whose *ALK* fusion were not identified by cfDNA, instead they were identified by the presence of the *ALK* resistance mutation. This reflects the complexity of fusion proteins and the fact that *ALK* has numerous fusion variants that may hinder identification by small fragment cfDNA analyses (53). Additionally, we are unable to estimate the true false negative rate of cfDNA in detecting *ALK* fusions given the database search parameters.

In conclusion, in the largest cohort of cfDNA *ALK* fusions reported to date, our data demonstrate that comprehensive cfDNA NGS testing is an additional tool that provides a noninvasive means of detecting targetable alterations in newly diagnosed patients, as well as resistance mutations and possible bypass pathways in patients progressing on targeted therapy. In this cohort, we were able to demonstrate the evolving and dynamic resistance profile in the longitudinally assessed patients with *ALK* fusions. We also describe *STRN–ALK* fusions as a potential emerging target upon progression in patients with *EGFR*-driven NSCLC. cfDNA provides a comprehensive view of the diversity and complexity of resistance mechanisms in a heterogeneous tumor cell population, highlighting the need to consider novel and combinatorial therapies in patients to help attenuate resistance.

Disclosure of Potential Conflicts of Interest

C.E. McCoach is a consultant/advisory board member for Guardant Health and Takeda. C.M. Blakely is a consultant/advisory board member for Jazz Pharmaceuticals, and reports receiving commercial research grants from Ignyta, MedImmune, Mirati, and Novartis. B. Levy is a consultant/advisory board member for AstraZeneca, Celgene, Eli Lilly, Genentech, Merck, and Takeda. A.T. Le has ownership interests (including patents) at Molecular Abbott. D.L. Aisner is a consultant/advisory board member for AbbVie and Bristol-Myers Squibb, and reports receiving commercial research grants from Genentech. R.B. Lanman has ownership interests (including patents) at Guardant Health. A.T. Shaw is a consultant/advisory board member for Ariad/Takeda, Genentech, Novartis, Pfizer, and TP Therapeutics. R.C. Doebele has ownership interests (including patents) at Rain Therapeutics, is a consultant/advisory board member for Ariad, AstraZeneca, Clovis Oncology, Ignyta, OncoMed, Pfizer, Spectrum Pharmaceuticals, Takeda, and TrovaGene, and reports receiving commercial research grants from Ignyta,

Loxo Oncology and Mirati Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.E. McCoach, C.M. Blakely, K.C. Banks, B. Levy, B.M. Chue, V.M. Raymond, S.N. Waqar, W.T. Purcell, D.L. Aisner, R.B. Lanman, A.T. Shaw, R.C. Doebele

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