original report

Tracking the Evolution of Resistance to ALK Tyrosine Kinase Inhibitors Through Longitudinal Analysis of Circulating Tumor DNA

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(continued)

abstract

Purpose ***ALK*** **(anaplastic lymphoma kinase) rearrangements predict for sensitivity to ALK tyrosine kinase inhibitors (TKIs); however, responses to ALK TKIs are generally short lived. Serial molecular analysis is an informative strategy used to identify genetic mediators of resistance. Although multiple studies support the clinical benefits of repeat tissue sampling, the clinical utility of longitudinal circulating tumor DNA analysis has not been established in ALK-positive lung cancer.**

Patients and Methods We used a 566-gene hybrid-capture next-generation sequencing assay to perform a longitudinal analysis of plasma specimens from 22 ALK-positive patients with acquired resistance to ALK TKIs to track the evolution of resistance during treatment. To determine tissue–plasma concordance, we compared plasma findings with the results of repeat biopsies.

Results At disease progression, we detected an *ALK* fusion in plasma from 19 (86%) of 22 patients and identified *ALK* resistance mutations in plasma specimens from 11 patients (50%). There was 100% agreement between tissue- and plasma-detected *ALK* fusions. Among 16 patients for which contemporaneous plasma and tissue specimens were available, we observed 100% concordance between *ALK* mutation calls. *ALK* mutations emerged and disappeared during treatment with sequential ALK TKIs, which suggests that plasma mutation profiles were dependent on the specific TKI administered. *ALK* G1202R—the most frequent plasma mutation detected after progression on a second-generation TKI—was consistently suppressed during treatment with lorlatinib.

Conclusion Plasma genotyping by next-generation sequencing is an effective method for detecting *ALK* fusions and *ALK* mutations in patients who experience disease progression on ALK TKIs. The correlation between plasma *ALK* mutations and the response to distinct ALK TKIs highlights the potential for plasma analysis to guide the selection of ALK-directed therapies.

INTRODUCTION

Oncogenic rearrangements that result in the constitutive activation of anaplastic lymphoma kinase (ALK) define a molecular subtype of non–small-lung cancer (NSCLC) that is characterized by sensitivity to ALK tyrosine kinase inhibitors (TKIs).[1,2] Since the identification of *ALK* rearrangements in NSCLC in 2007, four TKIs have become standard therapies for patients with advanced disease.[3-6] Although these TKIs have significantly improved clinical outcomes, most patients experience relapse within 1 to 2 years.[1] Despite a shared molecular driver, the magnitude of benefit from TKI treatment varies widely between patients. Repeat biopsies upon disease progression have been instrumental in elucidating the molecular mechanisms that drive resistance to ALK inhibitors, including the distinct spectrum of *ALK* mutations associated with resistance to each TKI[7]; however, repeat biopsies may be challenging to obtain and single-site sampling may not capture the spatial heterogeneity of resistance mechanisms.

Analysis of circulating tumor DNA (ctDNA) is an emerging approach for tumor genotyping.

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As plasma sampling is minimally invasive and ctDNA may be derived from all metastatic sites, longitudinal monitoring of genetic alterations in ctDNA may overcome many of the limitations of tissue sampling. Numerous studies have established digital polymerase chain reaction–based plasma genotyping as a reliable method for detecting mutations in patients with *EGFR*-mutant NSCLC[8,9]; however, it is challenging to perform multiplex testing and detect fusions using digital polymerase chain reaction.[10] In contrast, plasma genotyping by next-generation sequencing (NGS) can simultaneously interrogate multiple genes and identify a broad range of molecular alterations, including chromosomal rearrangements[10]; therefore, NGS may be optimally positioned to characterize TKI resistance in *ALK*-rearranged—that is, ALK-positive—NSCLC.

To date, NGS-based plasma genotyping studies have included only a small number of patients with ALK-positive NSCLC.[11-13] Here, we performed longitudinal ctDNA analysis with a hybrid capture–based NGS plasma genotyping platform to assess tissue–plasma concordance and study the evolution of resistance in patients who were treated with sequential ALK TKIs.

PATIENTS AND METHODS

Study Population

This study was conducted at Massachusetts General Hospital between June 2015 and January 2017. During this period, plasma was longitudinally collected from patients with metastatic, ALK-positive NSCLC who received treatment with ALK TKIs. The institutional review board at Massachusetts General Hospital approved this study. All study participants provided written informed consent.

Data Collection

Plasma Collection. Twenty milliliters of blood was collected from patients before initiation of treatment, approximately every 8 to 12 weeks, and at disease progression. As sampling coincided with clinic visits, the sampling interval varied between patients. As a result of the timing of the study initiation, we could not collect pretreatment plasma from five patients.

Clinical Data Collection. Medical records were reviewed to extract data on clinicopathologic features and treatment histories. Follow-up data for patients was obtained through January 2017. Determination of disease progression was based on investigator assessment of imaging studies and clinical status.

Molecular Analysis

Tumor Tissue. *ALK* rearrangements were identified by fluorescence in situ hybridization (FISH; n = 7) or NGS via Foundation One (n = 3), OncoPanel (n = 1), or a solid fusion assay (n = 1).[14-16] In the 10 remaining patients, ALK status was determined by the combination of FISH and NGS (n = 9) or FISH and immunohistochemistry (Ventana D5F3 antibody; n = 1).[17] SNaPshot NGS (Data Supplement) was employed to identify acquired genetic alterations, including *ALK* mutations.[14]

Plasma. DNA was extracted from frozen plasma specimens by using the QIAamp Circulating Nucleic Acid kit (Qiagen, Wetzlar, Germany). Libraries were constructed with Illumina TruSeq Nano DNA Library Prep kit (Illumina, San Diego, CA), enriched for a 566-gene Pan-Cancer gene panel (Data Supplement) by using Agilent SureSelect XT Custom baits (Agilent Technologies, Santa Clara, CA), and sequenced on an Illumina HiSEquation 2500 sequencer to a median of 105 million reads, which yielded a median coverage of 1,195×.[18] Sequence data were aligned to the hg19 reference genome. Variants were called with Pindel, Socrates, PureCN, and MuTect by using the default 0.5% lower threshold.[19-22] Variants were then annotated, restricted to nonsynonymous mutations that involved protein-coding regions, and filtered with the assistance of common reference databases and internal controls to remove germline variants and artifacts. All *ALK* resistance mutations were regenotyped with the Genome Analysis Toolkit (Broad Institute, Cambridge, MA). Those with at least four alternate reads were retained on the basis of a calculated background mutation rate and false-positive rate of 1^{e-4} and 1^{e-6}, respectively. The detection threshold for novel alterations in this pipeline was 1%. Called alterations were visually confirmed in Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

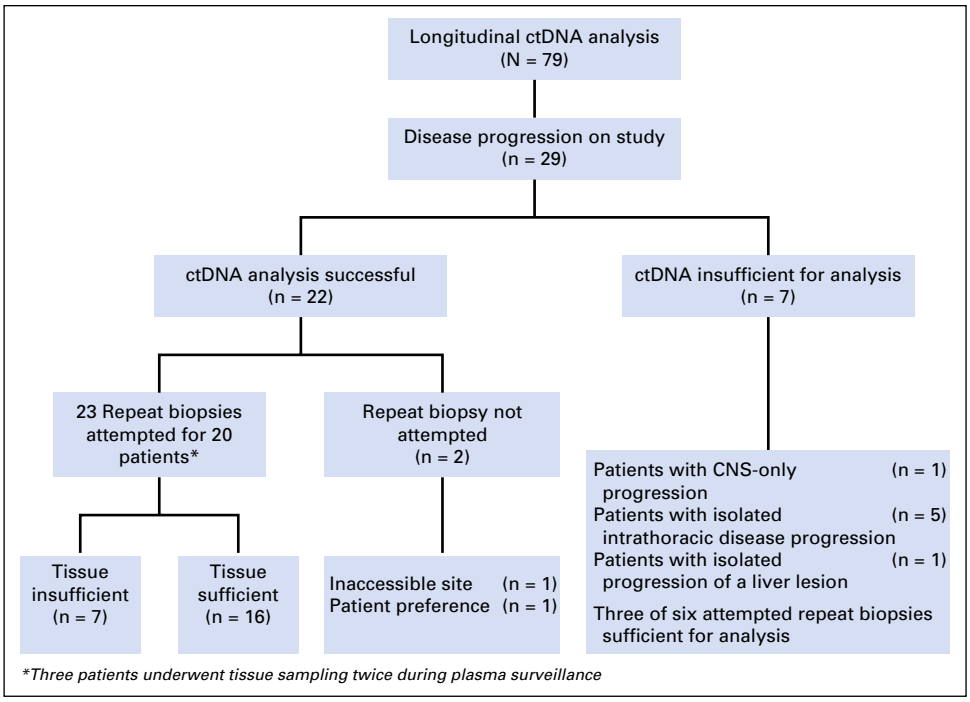

Fig 1. Study schema depicting the incidence of progression and success rates of tissue and plasma sampling among patients who experienced disease progression during the study period. Circulating tumor DNA (ctDNA) analysis successful indicates those samples for which tumor-derived mutations were successfully identified in the plasma assessment. ctDNA insufficient for analysis indicates those samples that were technically successful, but had insufficient tumor DNA present in the plasma to reliably estimate underlying tumor genotype.

RESULTS

Patient Characteristics

Plasma was longitudinally collected from 79 patients with metastatic, ALK-positive NSCLC (Fig 1). As this study was primarily focused on investigating the role of ctDNA analysis at disease progression, this report is limited to patients who had progressive disease and analyzable plasma specimens. Twenty-nine patients experienced disease progression during the study period. At the time of progression, we detected tumor DNA in plasma samples from 22 (76%) of 29 patients. Of the seven patients who did not have detectable ctDNA, one patient experienced CNS-only progression, five patients had intrathoracic progression, and one patient had liver oligoprogression (Fig 1). Baseline characteristics of the 22 patients and their treatment histories are summarized in Table 1, Figure 2, and the Data Supplement.

Plasma genotyping was performed on 88 plasma samples from 22 patients. We collected plasma at multiple time points during treatment with a single ALK TKI from eight patients and serial time points during treatment with two sequential ALK TKIs from 10 patients. For two patients, longitudinal sampling spanned treatment with three ALK TKIs. The temporal relationship between plasma collection and the treatments that were administered is depicted in the Data Supplement and Figure 2.

Concordance Between Tissue and Plasma Genotypes

We first evaluated concordance between tissue and plasma genotyping for *ALK* fusions, *ALK* mutations, and non-*ALK* alterations. Twenty (91%) of 22 patients underwent biopsy of a resistant site at relapse (Fig 1). Three patients underwent biopsies at two separate time points, with each biopsy representing disease progression on a distinct ALK TKI. Of 23 biopsies, tissue was adequate for NGS analysis in 16 specimens (70%) obtained from 14 patients. A biopsy was considered contemporaneous if performed within 3 weeks of plasma sampling. For two patients, tissue and plasma sampling were separated by approximately 6 weeks. As these two patients continued treatment with the same ALK TKI after experiencing progression, they were included in the analysis.

Fusion Concordance. At disease progression, an *ALK* fusion was detected in plasma from 19 (86%) of 22 patients. Of the three patients without a detectable *ALK* fusion, one patient experienced progression of intrapulmonary metastases, whereas the others experienced multisite progression (Data Supplement). We detected *EML4-ALK* in plasma from 18 patients

Table 1. Baseline Clinical and Pathologic Characteristics

Characteristic	All Patients (N = 22)
Age at diagnosis, years	
Median	52
Range	23-72
Sex, No. (%)	
Male	12 (55)
Female	10 (45)
Smoking history, No. (%)	
Never	14 (63)
Light (≤ 10 pack years)	5 (23)
Heavy (> 10 pack years)	3 (14)
Histology, No. (%)	
Adenocarcinoma	22 (100)
Stage at diagnosis, No. (%)	
I	0 (0)
II	2 (9)
III	2 (9)
IV	18 (82)
No. (%) of ALK TKIs before initial plasma collection*	
0	5 (23)
1	3 (13)
2	11 (50)
≥ 3	3 (13)

Abbreviation: ALK, anaplastic lymphoma kinase; TKI, tyrosine kinase inhibitor.
*The total number of ALK TKIs on which a patient had experienced progression before the collection of the initial plasma specimen. In instances where plasma was collected during sequential treatment with distinct ALK inhibitors, the line of therapy at initial plasma collection is represented.

(94%; Appendix Fig A1). Although we confirmed that an *ALK* fusion was present in the remaining patient, we were not able to determine the partner gene. Because tissue genotyping by FISH and immunohistochemistry does not identify the specific fusion partner or variant, we were unable to compare tissue and plasma fusions in eight patients. Among the 12 patients for whom the fusion partner was known and an *ALK* fusion was detected in the plasma, there was 100% agreement between tissue and plasma fusion calls (Fig 3A). Similarly, there was 100% concordance between tissue and plasma variant calls for the 11 patients who had an *EML4-ALK* variant that was identified by both plasma and tissue.

ALK Mutation Concordance. Point mutations in the *ALK* kinase domain were identified in plasma specimens from 11 patients (50%), five of whom had paired biopsies at the time of mutation detection (Fig 3B). In eight of 11 patients, *ALK* mutations were considered sufficient to mediate TKI resistance. In the remaining three patients, *ALK* mutations were detected during progression on lorlatinib, a pan-inhibitory ALK TKI that was expected to be active against a broad spectrum of mutations. *ALK* G1202R—present in the plasma from six patients—was the most common *ALK* mutation. Three patients had multiple *ALK* mutations.

All tissue-detected *ALK* mutations were also identified in corresponding plasma samples, and all plasma-detected *ALK* mutations were also present in paired biopsies. Overall, there was 100% concordance between detected tissue and plasma *ALK* mutations when temporally correlated specimens were assessed. Among 17 patients who had plasma collected during progression on a second-generation ALK inhibitor—that is, ceritinib, alectinib, or brigatinib—plasma *ALK* mutations were identified in eight patients (47%; Data Supplement), including I1171N (n = 1), G1202R (n = 5), I1171N/G1202R (n = 1), and E1210K (n = 1). The observed frequency of plasma *ALK* mutations is consistent with our previous analysis of resistance biopsies from patients who developed resistance to second-generation inhibitors, which included five patients from the current plasma cohort.[7]

Non-ALK Alterations. As we did not detect any differences in tissue alterations that were identified in serial specimens from the three patients who underwent multiple biopsies, we restricted the concordance assessment to one biopsy per individual. Among these 14 biopsies, we identified mutations that involved genes other than *ALK* in seven specimens (50%). *TP53* was mutated in five resistance biopsies (36%). For three of these patients, *TP53* mutation was present at the initial diagnosis. At progression, we detected a *TP53* I195T mutation in a biopsy that was not detected in plasma from MGH087. Similarly, we only detected *MORC* I511V and *WDFY4* T1039N mutations in MGH919's tissue. All other tissue mutations were also present in plasma (Data Supplement). We did not detect *KRAS* or *EGFR* mutations in plasma or tissue specimens.

We also interrogated plasma for copy number alterations. We identified high-level amplification of *BCL2L1* and *GNAS* (> 20 copies each) in the plasma of MGH919 during treatment

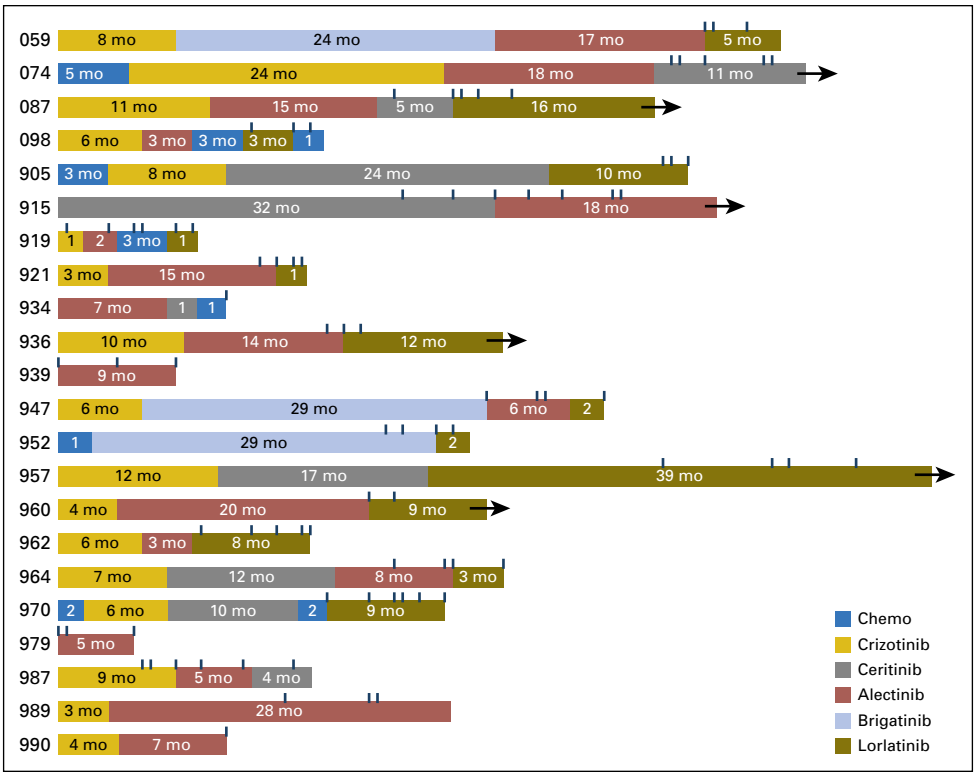

Fig 2. Temporal relationship between plasma collections and administered treatments. Dark blue hash marks above boxes indicate the timing of the plasma collection. Arrows indicate that the patient continued on treatment after plasma collection. Note that some patients continued treatment beyond experiencing progression.

with lorlatinib (Data Supplement). Although we were not able to obtain tissue after the patient had developed progression on lorlatinib, amplification was not present when a prelorlatinib liver biopsy was assessed using the same 566-gene panel. We identified one case (MGH939) of *MET* amplification in an alectinib-resistant lung biopsy specimen. Despite high-level *MET* amplification (> 25 copies) in tissue, we did not detect *MET* amplification in the corresponding plasma specimen; however, as the maximum allelic frequency (AF) of detected alterations in the plasma of MGH939 was approximately 2%, the degree of amplification was below the limit of detection of the assay.

Evolution of Resistance During Molecular Surveillance

ALK Fusion Kinetics and Correlation With Disease Status. We hypothesized that the trend in plasma *ALK* fusion AF for each patient would correlate with disease status. Across patients, we observed a wide range of fusion AF in patients who had developed resistance to TKI treatment. Furthermore, the absolute change in fusion AF was modest for many patients, with peak progression values of < 0.5%. Still, changes in fusion AF reflected clinical status, with higher AF observed at disease progression than during response.

Evolution of ALK Resistance Mutations During Therapy. Preclinical models and case reports suggest that the presence of a specific *ALK* mutation in a TKI-resistant tissue specimen may predict response to subsequent ALK TKIs[1,7,23]; therefore, we analyzed plasma specimens from patients with *ALK* mutations who received treatment with additional ALK TKIs to evaluate the potential of using plasma findings to inform sequential therapies. As noted above, we considered *ALK* mutations to be the major driver of resistance for eight (36%) of 22 patients in our cohort. During the follow-up period, seven of these patients (88%) went on to receive treatment with a TKI that targeted the *ALK* mutation (Data Supplement). In all cases, we observed the suppression of pretreatment plasma *ALK* mutations when a subsequent ALK TKI with preclinical activity against the mutation was initiated (Data Supplement). For example, MGH987 developed a plasma *ALK* I1171N mutation on alectinib treatment that was not present during treatment with crizotinib. On the basis of studies that support its activity against *ALK* 11171N,[23] ceritinib was initiated, which led to a radiographic and molecular response with a decrease

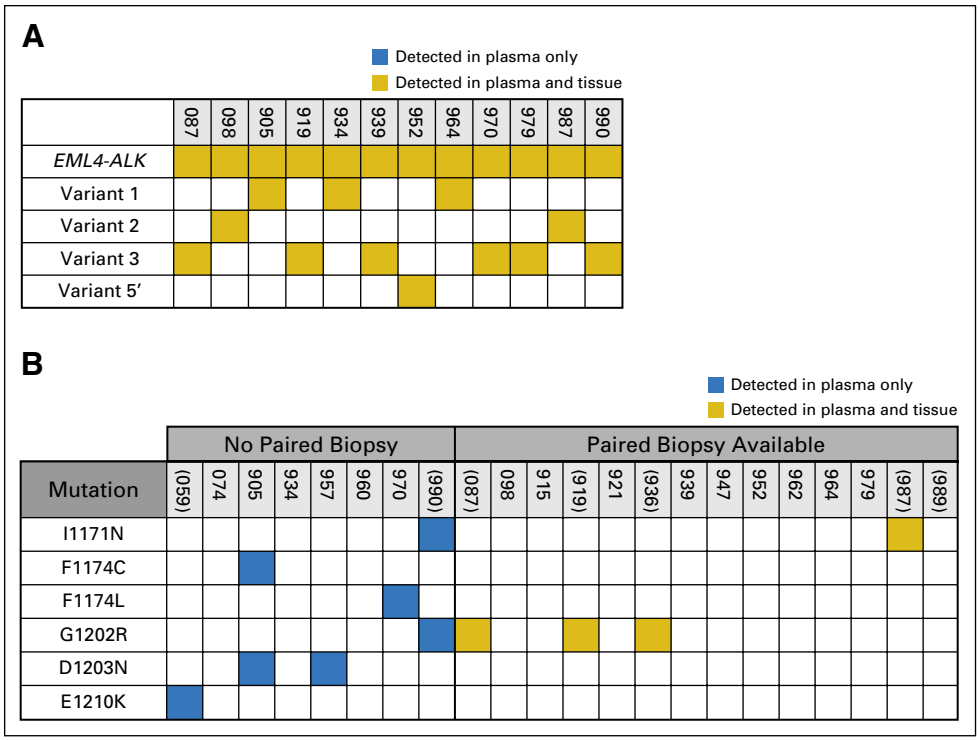

Fig 3. Tissue–plasma concordance analysis. Gold box indicates that an alteration was detected in both plasma and tissue; blue box indicates that an alteration was present in the plasma only. (A) Concordance between fusion calls. In instances where the *ALK* fusion partner was not specified during tissue genotyping, patients were excluded from the concordance analysis. The fusion variant in MGH905's plasma was not identified by tissue analysis.(B) Concordance between *ALK* (anaplastic lymphoma kinase) mutation calls. Parentheses denote patients for whom the *ALK* mutation was considered the dominant resistance mechanism. For patients without paired biopsies, *ALK* mutations that were detected at the time of progression on an ALK tyrosine kinase inhibitor are listed. None of the patients without paired biopsies had plasma collected during progression on sequential ALK inhibitors. *ALK* mutations in the plasma of MGH905 occurred in cis. Because the mutations were separated by too many bases, the allelic relationship (cis *v* trans) could not be determined for MGH990's *ALK* mutation.

in *ALK* I1171N AF from 4.5% to undetectable after 6 weeks (Fig 4A). Similarly, we detected an E1210K mutation in the plasma of MGH059 after experiencing progression on alectinib, which became undetectable during response to lorlatinib, a next-generation ALK TKI with preclinical activity against this mutation[7] (Appendix Fig A2, and Data Supplement).

Among *ALK* resistance mutations that were identified in patients who experienced progression on second-generation ALK TKIs, the solvent front G1202R mutation is the most common.[7] Of note, lorlatinib has been shown to retain activity against cancers that harbor G1202R.[7,24] In our cohort of patients, six (35%) of 17 patients who had plasma collected during treatment with a second-generation ALK TKI acquired the G1202R mutation when experiencing disease progression. One patient (MGH989) underwent microwave ablation to treat a liver lesion with a biopsy-proven G1202R mutation and continued treatment with alectinib for an additional 6 months. After ablation, G1202R AF decreased from 4% to undetectable (Fig 4C). The remaining five patients were treated with lorlatinib. Two of these patients—MGH087, depicted in Figure 4B and MGH936, depicted in Appendix Figure A2—achieved a response to treatment and plasma clearance of G1202R. MGH990 had a radiographic response to treatment, but we could not evaluate the molecular response as we were only able to collect a single plasma specimen.

Although the G1202R mutation was suppressed in plasma during the treatment with lorlatinib, two of the four patients with plasma-detected G1202R developed progressive disease soon after initiating lorlatinib (Appendix Fig A3). MGH947 experienced symptomatic progression of a brain metastasis, which prompted a craniotomy. G1202R was not detected in the resected lesion. MGH919 developed multisite progression and rapidly declined. We were not able to obtain a biopsy, but plasma analysis demonstrated an increase in copy number of *GNAS* and *BCL2L1* and a marked increase in the AF of mutations that involved other genes during treatment (Fig 5). In both cases, *EML4-ALK* fusion AF increased at progression despite the suppression of *ALK* mutations, which supports the notion that resistance was independent of genetic *ALK* alterations.

DISCUSSION

Despite a shared molecular driver, the clinical course is variable for patients with ALK-positive NSCLC treated with ALK TKIs. Personalized

treatment approaches currently rely on serial tissue sampling to guide subsequent management; however, tissue sampling often fails to capture the dynamic and heterogeneous nature of resistance. To our knowledge, we present here the largest study of longitudinal plasma genotyping in ALK-positive NSCLC to date. Our findings highlight the potential clinical utility of hybrid-capture NGS for improving our understanding of the molecular drivers of resistance.

In our cohort of patients, 76% of plasma samples contained sufficient tumor-derived DNA for molecular analysis compared with 65% of biopsy specimens, which confirms that both are reliable approaches. We identified plasma *ALK* mutations in one half of our patients at disease progression, many of whom had been exposed to a second-generation ALK inhibitor. Overall, there was a high degree of concordance between plasma and tissue alterations. In addition to demonstrating agreement between plasma and tissue at specific time points, we observed changes in plasma *ALK* mutation status and mutation AF during longitudinal analysis. These results highlight the advantages of noninvasive genotyping and the potential of serial plasma analysis to elucidate the impact of therapeutic selective pressure on clonal dynamics. Furthermore, our observation that the presence of specific plasma *ALK* mutations correlated with the response and resistance to distinct ALK TKIs lends support to the emerging practice of using *ALK* mutations to inform the selection of ALK TKIs.

Although tissue sampling is the gold standard for molecular analysis, sampling a single site of disease may overestimate the contribution of a particular alteration to the resistant phenotype. For example, we detected an *ALK* G1202R mutation at 50% AF in a progressing liver lesion, which suggested that it was a major contributor to alectinib resistance in the case of MGH919. In contrast, the plasma G1202R AF of 1% was less than the AF of other plasma-detected molecular alterations, including multiple non-*ALK* alterations (Fig 5). The patient's primary disease progression on lorlatinib supports the conclusion that *ALK* G1202R was likely not the major driver of resistance. Moreover, this case reinforces the notion that the analysis of liquid biopsies may be more informative than tissue-based genotyping in certain situations.

To date, the study of resistance to ALK therapeutics has primarily focused on *ALK* mutations. As a significant proportion of patients may not acquire *ALK* mutations, we evaluated whether tracking plasma fusion AF might be used to anticipate disease progression. Our results indicate that even when fusions can be detected longitudinally, the dynamic range of the fusion AF

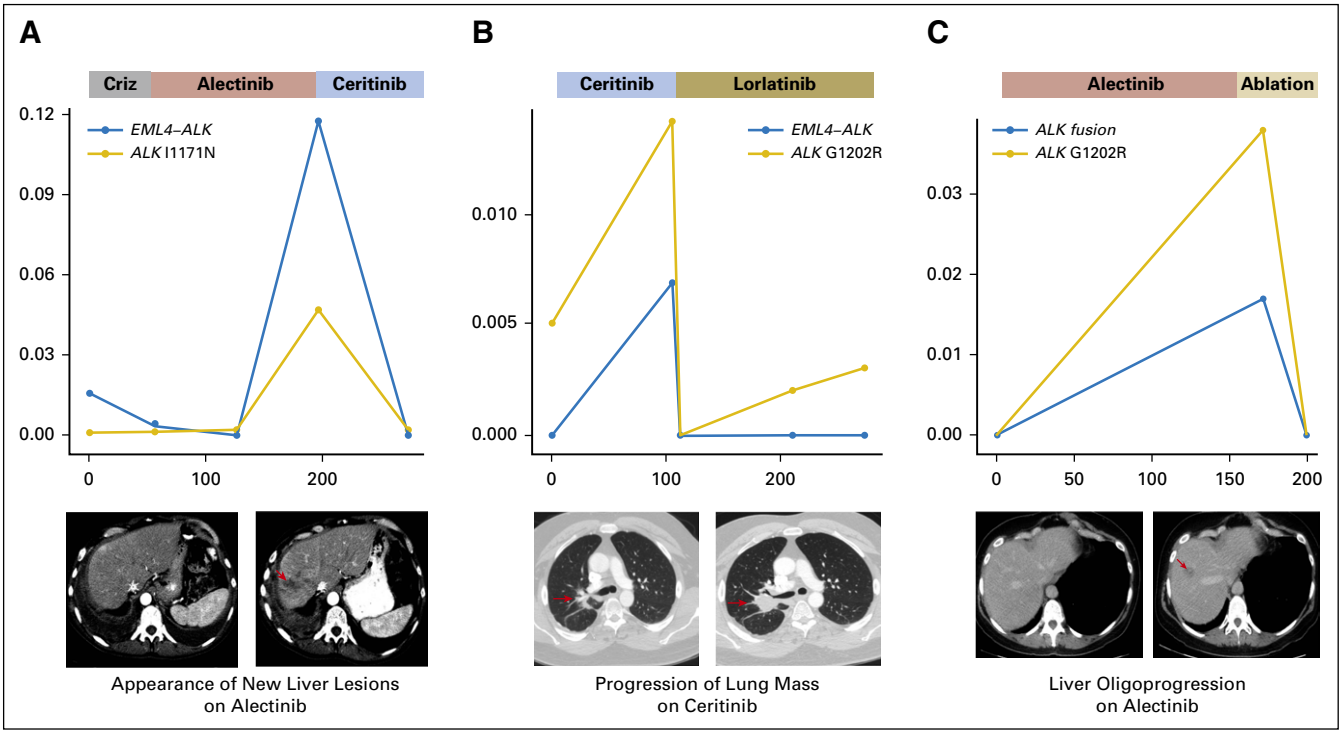

Fig 4. Plasma *ALK* (anaplastic lymphoma kinase) mutation kinetics during treatment. Figure illustrates the change in the allelic fraction of *EML4-ALK* and *ALK* mutations during sequential treatment with next-generation ALK inhibitors for (A) MGH987 and (B) MGH087. (C) *ALK* fusion and mutation kinetics before and after ablation of an oligometastasis (MGH989). The *x*-axis depicts days from initial plasma collection, whereas the *y*-axis reports allelic fraction. Criz, crizotinib.

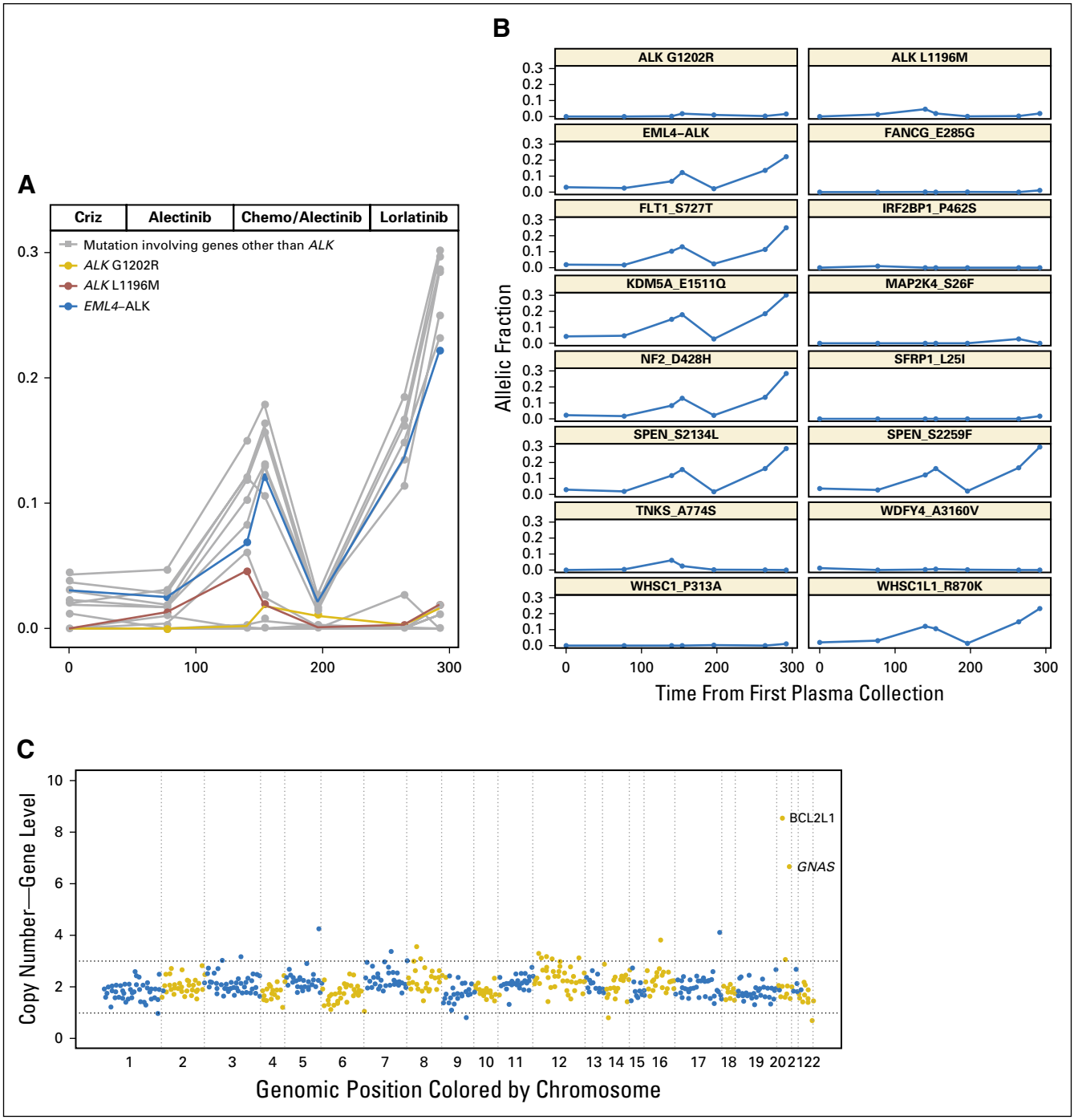

Fig 5. Longitudinal analysis of the plasma of MGH919. (A) Allelic fraction of *ALK* (anaplastic lymphoma kinase) fusion (blue), *ALK* mutations (red and gold), and non-*ALK* alterations (gray) during treatment with sequential ALK inhibitors. The *x*-axis represents days from initial plasma collection, whereas the *y*-axis represents allelic fraction. (B) Individual mutation kinetics during treatment. (C) Plasma copy number plot from a specimen collected during treatment with lorlatinib shows focal amplification of *GNAS* and *BCL2L1*. *ALK* L1196M and G1202R mutations were in trans. Chemo, chemotherapy; Criz, crizotinib.

during response and progression may be limited. These findings suggest that trending plasma *ALK* fusions as a surrogate for disease status may not be uniformly applicable. Indeed, we did not detect an *ALK* fusion in the plasma from three of our patients during disease progression. The ratio of the resistance mutation AF to the activating mutation AF can offer insight into tumor heterogeneity and predict clinical outcomes in patients with *EGFR*-mutant NSCLC[8,25]; however, the differing efficiencies of capturing point mutations and structural variants may result in

an *ALK* mutation AF that exceeds fusion AF, which suggests that similar calculations may be challenging for ALK-positive NSCLC. Despite these shortcomings, our data suggest that the quantitative assessment of structural variants may be clinically useful and complementary to radiographic assessment in some patients.

Overall, our data confirm that plasma genotyping by using hybrid-capture NGS technology can reliably detect *ALK* fusions and *ALK* resistance mutations in patients with ALK-positive NSCLC; however, there are several limitations of this study, including the small size of our cohort, inconsistent sampling intervals that led to variation in the duration of follow-up after and before disease progression, and the inability to obtain pretreatment plasma and paired biopsies for all patients. Furthermore, as a result of the small contribution of tumor DNA to total cell-free DNA in most patients, we cannot definitively exclude the presence of undetected amplification events at resistance. Finally, as the assay only analyzed genetic alterations in 566 genes, the current analysis does not account for resistance as a result of alterations in genes not covered by the panel and nongenetic mediators of drug resistance.

The optimal strategy for integrating plasma genotyping into clinical practice remains to be established. For patients with *EGFR*-mutant NSCLC, there are two US Food and Drug Administration–approved liquid biopsies that may be used to select initial and subsequent targeted therapies. As identifying *ALK* resistance mutations is emerging as an important consideration for the management of ALK-positive NSCLC, we anticipate that genotyping plasma to characterize resistance to ALK TKIs will also become a routine part of patient care. Although tissue remains the gold standard, larger ALK-specific concordance studies may ultimately pave the way for ctDNA analysis to become an essential component of the evaluation of ALK TKI resistance. Beyond identifying alterations at resistance, one of the major advantages of plasma genotyping is its ability to track response kinetics throughout the treatment course. Additional studies are indicated to determine whether plasma mutation clearance and subclinical molecular relapse are predictive of clinical outcomes.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Other Relationship: Third Rock Ventures

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No relationship to disclose

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Stock and Other Ownership Interests: Novartis

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Research Funding: Pfizer, Novartis, Genentech

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Consulting or Advisory Role: Novartis, Boehringer Ingelheim, Clovis Oncology, Genentech, Bristol-Myers Squibb, Theravance, Loxo

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Appendix

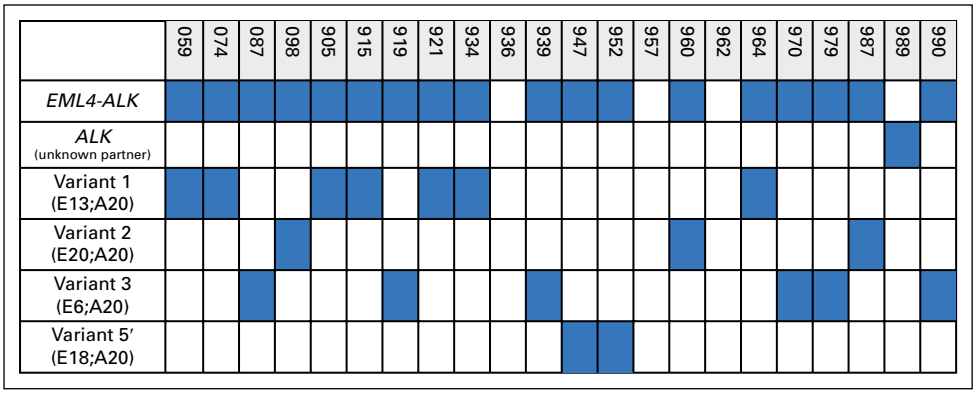

Fig A1. *ALK* (anaplastic lymphoma kinase) fusions detected in plasma. Figure illustrates the spectrum of *ALK* fusion variants that were detected in plasma specimens. Blue indicates the presence of a fusion or fusion variant, whereas white indicates the absence of a detectable fusion. Numbers denote the exon breakpoints for each gene. In two of the three patients for whom plasma analysis failed to detect a fusion, an *EML4-ALK* fusion (variants 3 and 5) was identified in matching tissue. The remaining patient did not undergo a repeat biopsy, but was ALK positive by fluorescence in situ hybridization at diagnosis. E, *EML4*; A, *ALK*.

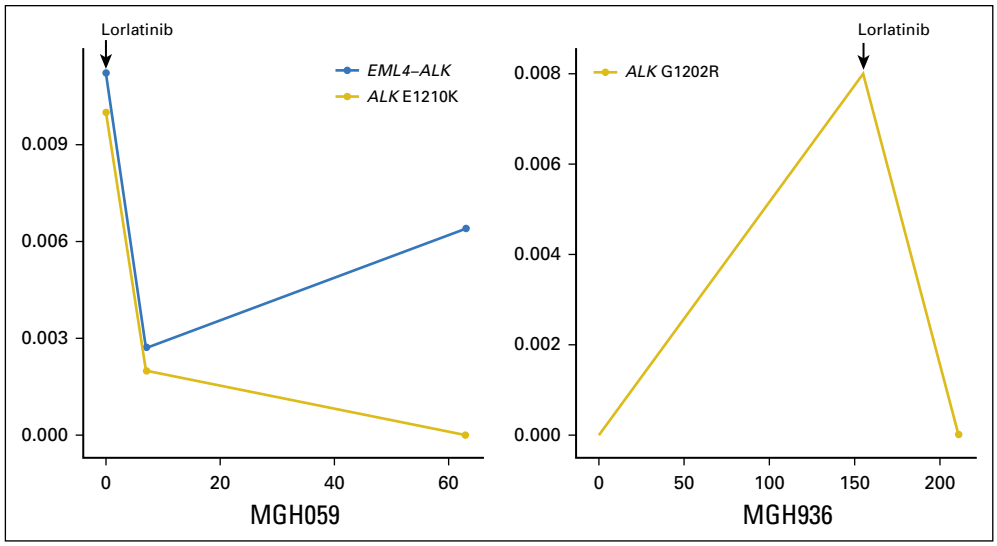

Fig A2. Longitudinal monitoring during sequential treatment with ALK (anaplastic lymphoma kinase) inhibitors. Figure illustrates *ALK* fusion (MGH059 only; left) and *ALK* mutation plasma allelic fraction during progression on alectinib and the subsequent response to lorlatinib. The assay did not detect an *ALK* fusion in the plasma of MGH936 during longitudinal assessment. The *y*-axis indicates the allelic fraction of the *ALK* alterations.

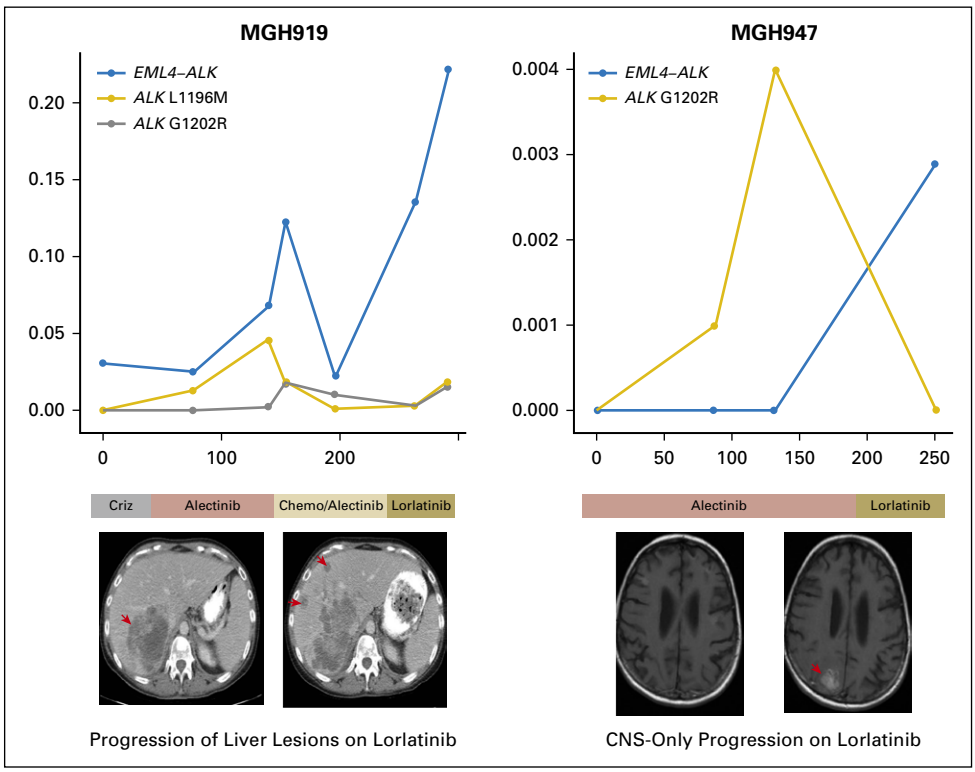

Fig A3. Plasma *ALK* (anaplastic lymphoma kinase) fusion and *ALK* mutation allelic fractions of MGH919 (left) and MGH947 (right) during treatment with ALK inhibitors. The *y*-axis indicates the allelic fraction of the *ALK* fusion and *ALK* mutations. Chemo, chemotherapy; Criz, crizotinib.