HHS Public Access

Author manuscript

Lung Cancer. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Lung Cancer. 2016 September ; 99: 17–22. doi:10.1016/j.lungcan.2016.06.006.

De novo *ALK* kinase domain mutations are uncommon in kinase inhibitor-naïve *ALK* rearranged lung cancers

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Abstract

Introduction—Anaplastic lymphoma kinase (*ALK*) rearranged lung adenocarcinomas are responsive to the multitargeted ALK inhibitor crizotinib. One of the common mechanisms of resistance to crizotinib is the acquisition of *ALK* kinase domain mutations. However, the presence of *ALK* mutations in crizotinib-naïve tumors has not been widely reported and it is unclear if *de novo ALK* mutations affect the response to crizotinib.

Methods—We analyzed preclinical models of *ALK* rearranged lung cancers that were sensitive/resistant to ALK inhibitors, probed our institutional and other lung cancer databases for tumors with *ALK* kinase domain mutations, and evaluated tumor response to crizotinib.

Results—*ALK* rearranged cell lines with *ALK* kinase domain mutations were heterogeneously less inhibited by increasing concentrations of crizotinib than cells driven solely by EML4-ALK fusions. Previous *ALK* rearranged lung cancer cohorts did not report *ALK* kinase mutations in

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CONFLICT OF INTEREST STATEMENT

Daniel B. Costa has received consulting fees and honoraria from Pfizer Inc (unrelated to the current work), consulting fees from Ariad Pharmaceuticals (unrelated to the current work), and honoraria from Boehringer Ingelheim (unrelated to the current work). Antonio R. Lucena-Araujo, Jason P. Moran, Paul A. VanderLaan, Dora Dias-Santagata, Erik Folch, Adnan Majid, Michael S. Kent, Sidharta P. Gangadharan, Deepa Rangachari, Mark S. Huberman, and Susumu S. Kobayashi have no conflicts to disclose. No other conflict of interest is stated.

inhibitor-naïve tumors. We identified one TKI-naïve *ALK* rearranged tumor with an *ALK* kinase domain mutation: *ALK*-S1206F (mutations at ALK-S1206 shifted crizotinib inhibitory curves only minimally in preclinical models). The never smoker woman whose tumor harbored *de novo EML4-ALK*-E5;A20+*ALK*-S1206F achieved radiographic response to crizotinib 250mg twice daily.

Conclusions—Combining data from our and prior cohorts, *ALK* kinase domain mutations were uncommon events (<3% of cases) in ALK inhibitor-naïve *ALK* rearranged lung adenocarcinomas but their effect on intrinsic resistance to ALK inhibitors should be better evaluated.

Keywords

mutation; lung cancer; adenocarcinoma; ALK; kinase domain; crizotinib

INTRODUCTION

Anaplastic lymphoma kinase (*ALK*) rearrangements lead to oncogene addiction in around 5% of lung adenocarcinomas [1–3]. In preclinical models, ALK fusion proteins retain the tyrosine kinase domain (amino acid [aa] 1116 to 1383, encompassing exons 22–25) of the kinase that in turns engages proliferative and antiapoptotic downstream targets of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) and phosphatidylinositol-3-kinases (PI3K)/protein kinase B (AKT) cascades [2]. Multitargeted ALK tyrosine kinase inhibitors (TKIs) are capable of inhibiting these intricate signaling networks to induce antiproliferative and proapoptotic effects [4, 5]. In the clinic, the multitargeted TKI crizotinib induces tumor regression in almost all cases of advanced *ALK* rearranged non-small-cell lung cancer (NSCLC) for a period of time before the tumor acquires resistance to monotherapy [1, 6]. The most common mechanisms of acquired resistance to crizotinib in preclinical models and clinical specimens include: *ALK* kinase domain mutations that shift the sensitivity profile of crizotinib [7], activation of other oncogenes (such as epidermal growth factor receptor [EGFR]) that lead to signaling bypass tracks [8], and inadequate pharmacokinetic exposure [9]. ALK kinase domain mutations - including *ALK*-1151Tins, L1196M, G1202R and G1269A - when present with EML4-ALK fusion proteins heterogeneously change the pattern of sensitivity to different ALK TKIs; with some resistant and others sensitive to the more potent inhibitors ceritinib, alectinib, brigatinib and lorlatinib [3, 10].

The presence of *de novo ALK* kinase domain mutations concurrently with *ALK* rearrangements in ALK TKI-naïve NSCLCs has not been reported previously by Thoracic Oncology multidisciplinary groups [7, 11, 12] or The Cancer Genome Atlas (TCGA) database [13]; and it is therefore unclear if *de novo ALK* mutations affect the response to evidence-based crizotinib in patients. Herein, we report the uncommon but present occurrence of *de novo* ALK kinase domain mutations in our institutional database of *ALK* rearranged NSCLCs and evaluate preclinical models to correlate with tumor response to crizotinib.

METHODS

Cell culture, proliferation assays and reagents

NCI-H3122 (H3122) cells, harboring *EML4-ALK*-E13;A20, were obtained as previously described [4, 5]. Variants of H3122 to model mechanisms of resistance to crizotinib, including H3122 CR1 (with *ALK*-L1196M provided by Dr. Jeffrey Engelman) and H3122 CR_A (with EGFR bypass track), were derived after long term exposure to crizotinib, as previously reported [7, 8]. All cells were maintained in RPMI-1640 medium (Mediatech, Manassas, VA) appended with one tenth fetal bovine serum and grown in usual conditions [14, 15].

Reagents

Crizotinib and afatinib were purchased from LC Laboratories (Woburn, MA). Ceritinib was purchased from Active Biochemicals (Hong Kong). All reagents were dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C.

Proliferation assay, western blot and antibodies

Selected cells were overlaid in 96-well plates, allowed to attach and then treated with or without TKIs (crizotinib, certinib or afatinib) for prespecified time points. Cell viability was determined by CellTiter 96 Aqueous One solution proliferation assay (Promega, Madison, WI). Experiments were performed in triplicate. Inhibitory proliferation curves and the 50% inhibitory concentration (IC_{50}) were made using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). To highlight in an isogenic system different ALK kinase domain mutants' effects on EML4-ALK, we recycled previously published IC_{50} values obtained using modified Ba/F3 cells from Friboulet et al [10]. H3122, H3122 CR1 and H3122 CR_A were lysed after exposure to TKIs or DMSO, lysates were separated, transferred to membranes and analyzed as previously described [8, 16]. AKT, phospho-AKT (pS473), phospho-ERK1/2 (pT202/pY204), phospho-ALK (pY1604) and ALK were purchased from Cell Signaling Technology (Beverly, MA). EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK1/2 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Primary antibodies were diluted 1:1000 and their recommended secondary antibodies diluted 1:10000.

Tumor and data collection

Patient-tumor pairs followed at the Thoracic Oncology multidisciplinary clinic at Beth Israel Deaconess Medical Center (BIDMC) with a diagnosis of NSCLC were registered through an ongoing Institutional Review Board-approved study [17–19]. Pathologic data, tumor genotype and radiographic images were assembled from retrospective chart extraction. Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was utilized. Data was collected and managed using the REDCap electronic data capture held at BIDMC. Additional published *ALK* rearranged NSCLC cohorts were identified by literature review [7, 11, 12]. In addition, the 2014 TCGA lung adenocarcinoma mutation database [13] was reviewed and collated for *ALK* genotypes and co-existing mutations using cBioPortal (http://www.cbioportal.org/index.do).

ALK fusion partner and kinase domain analysis

ALK fluorescence in situ hybridization (FISH) using the Vysis break apart probe set, as detailed previously [18], was our institution's screening test for *ALK* rearrangements. RNA was isolated from tumor tissue for evaluation of fusion partners using PCR-based or next generation sequencing assays, while DNA was isolated to sequence exons corresponding to the kinase domain of *ALK* (exons 21 to 27), as previously reported [16, 17, 20, 21].

RESULTS

Preclinical models of *ALK* rearranged NSCLC and resistance to crizotinib

We selected one cell line (H3122 with *EML4-ALK*-E13;A20) to mimic sensitivity to ALK TKIs and two representative cell lines (H3122 CR1 and H3122 CR_A) to mirror crizotinib resistance. H3122 had the lowest nanomolar IC_{50} values of dose-dependent proliferation experiments for crizotinib; while H3122 CR1 (with the *ALK*-L1196M mutation as the resistance mechanism) and H3122 CR_A (with EGFR bypass as the resistance mechanism) were not inhibited by clinically achievable levels [22] of crizotinib (Fig. 1A). Intracellular signaling analysis indicated that ALK TKIs suppressed phosphorylation of ALK, AKT and ERK in H3122 (Fig. 1B). Crizotinib was unable to inhibit phosphorylation of ALK, AKT or ERK in H3122 CR1, whereas it inhibited ALK in H3122 CR_A but enhanced EGFR levels continued to drive AKT and ERK (Fig. 1B).

To further confirm the aforementioned mechanisms of resistance to crizotinib, we were able to show that the more potent ALK TKI ceritinib was able to inhibit the proliferation of H3122 CR1 but not of H3122 CR_A (Fig. 1C). Co-inhibition of ALK and EGFR with ceritinib and afatinib, respectively, led to antiprofilerative effects at low ceritinib doses in H3122 CR_A (Fig. 1D).

These preclinical models confirmed that *ALK* kinase domain mutations (such as *ALK*-L1196M) can induce resistance to crizotinib and led us to question if *de novo ALK* mutations could be present in TKI-naïve ALK rearranged NSCLCs and/or could lead to primary insensitivity to crizotinib.

Tumors with *ALK* rearrangements and *de novo ALK* kinase domain mutations

To evaluate the spectrum of genomic *ALK* changes in NSCLC, we first turned to the TCGA 2014 cohort of 230 surgically resected lung adenocarcinomas [13]. The frequency of *ALK* rearrangements was 1.3% (none co-occurring with *ALK* mutations) and of any type of *ALK* exon mutation 6.5% (Fig. 2A). Interestingly, most mutations seems to be "passenger events" (e.g., outside the catalytic kinase domain) and only one sample (without co-occurring *ALK* rearrangement) had an *ALK* kinase domain mutation, and the mutation reported was an *ALK*-E1299* that would be considered inactivating/non-functional (Fig. 2B). Our BIDMC NSCLC cohort identified *ALK* rearrangements in 5.9% (41/690) of TKI-naïve cases screened by *ALK* FISH, and detailed information on fusion partners plus *ALK* kinase domain sequence was available for 6 tumors (Fig. 2B). One case with *EML4-ALK*-E5;A20 also harbored a *de novo ALK*-S1206F mutation.

To further expand the aforementioned results, we collated data from three published cohorts of advanced *ALK* rearranged NSCLCs in which information was available in crizotinib-naïve and –resistant biopsy specimens. None of these studies reported *de novo* ALK kinase domain mutations, and combining all cohorts one can come up with a frequency of only 2.7% (1/37) for *ALK* kinase mutations (Fig. 2C) in TKI-naïve *ALK* rearranged NSCLC (Fig. 2C). The latter numbers contrast with the combined frequency of 27.5% of activating *ALK* kinase domain mutations in crizotinib-resistant rebiopsy samples (Fig. 2D; p=0.0035 compared to TKI-naïve, Fisher's exact test).

Preclinical evaluation of *ALK* kinase domain mutations

We were curious to understand the extent of heterogeneity between different types of ALK kinase domain mutant proteins against crizotinib in the background of EML4-ALK. We identified a previously published report, from Friboulet et al [10], with a comprehensive set of Ba/F3 cell lines driven by EML4-ALK-E13;A20 and a variety of patient-identified ALK kinase domain mutants treated with crizotinib (Fig. 3A).

Of interest, the ALK mutant at position S1206 (ALK-S1206Y) showed the least fold inhibition of crizotinib IC_{50} when compared to EML4-ALK (Fig. 3A), raising the question if this mutant coexisting with EML4-ALK would or would not lead to clinical resistance in a patient in which crizotinib was given at the initial prescribed dose of 250mg twice daily [3, 9]. Whereas other ALK mutants (such as ALK-G1202R, L1196M, 1151Tins) had IC_{50}s >10 fold higher than EML4-ALK-E13;A20, and would be expected to lead to resistance to achievable concentrations of crizotinib (Fig. 3A).

Radiographic response to crizotinib in lung adenocarcinoma harboring *de novo EML4-ALK*-E5;A20+*ALK*-S1206F

The patient with *de novo EML4-ALK*-E5;A20+*ALK*-S1206F mutated lung adenocarcinoma was a 69 year-old never smoker woman who presented with pulmonary, pleural, nodal, osseous and hepatic metastases (Fig. 3B). The diagnostic specimen analyzed was a pleural effusion cellblock with 60% tumor content. 47% of cells were *ALK* FISH positive [nuc_ish (3′ALKx2~4,5′ALKx1~2)(3′ALKcon5′ALKx1~2)-(94/200)], the allele frequency of *ALK*-S1206F was 28% (i.e., one mutant allele per tumor cell) and *TP53*-R175C was additionally identified. The patient was prescribed crizotinib at 250mg twice daily and tolerated therapy without significant gastro-intestinal, visual or laboratorial adverse events. Within one week of therapy her clinical condition improved and imaging studies at the one month mark of crizotinib therapy disclosed remarkable decrease in tumor burden that was maintained at the two month mark (Fig. 3B), compatible with partial response by RECISTv1.1 with 30.3% decrease in target lesions. The patient continues on therapy at time of this report (month four of therapy).

The clinical/radiographic response suggests that the ALK-S1206F mutant, similar to ALK-S1206Y (Fig. 3A), may be fully inhibited in the background of EML4-ALK by initial plasma and tumor concentrations achieved by crizotinib 250mg twice daily *in vivo* [22].

DISCUSSION

The use of crizotinib and other multitargeted ALK TKIs has revolutionized the care of *ALK* rearranged NSCLCs; however, acquired resistance usually ensues after a period of 6–24 months [3]. ALK kinase domain mutations have been recognized in a portion of rebiopsy specimens upon progression on crizotinib, ceritinib, alectinib and lorlatinib [7, 10–12, 23]. Different mutations have divergent patterns of sensitivity to ALK TKIs in preclinical models. As examples, EML4-ALK with ALK-G1202R leads to inhibitory curves that suggest resistance to crizotinib, ceritinib, alectinib and brigatinib, but sensitivity to lorlatinib [10, 23]; while EML4-ALK with ALK-L1196M leads to resistance only to crizotinib and sensitivity to other aforementioned ALK TKIs [7, 10]. Approximately one third, as confirmed in this report (Fig. 1C), of ALK-driven tumors at time of acquired resistance to crizotinib display ALK kinase domain mutations while the same frequencies are unclear for other ALK TKIs. NSCLC cells can modulate p-glycoprotein expression as a mechanisms of systemic pharmacokinetic resistance to crizotinib [24], and this observation raises concern that crizotinib concentrations may decrease linearly over time within the cancer cell to a level where even “less resistant” ALK kinase mutants can exert inhibitory effects.

Our group questioned if *de novo ALK* kinase domain mutations co-occur with EML4-ALK and if these compound genomic changes explain some of the rare cases of primary insensitivity of ALK-driven NSCLCs to crizotinib. The data presented here suggest that *de novo ALK* kinase domain mutations are uncommon in tumors with *ALK* rearrangements (<3% of cases) and even less common in NSCLCs in general (<1% of cases). Nonetheless, the only TKI-naïve case with co-occurring *EML4-ALK*-E5;A20 plus *ALK*-S1206F was quite instructive and shows how clinical-level data can complement preclinical systems. The patient received crizotinib 250mg twice daily and had a rapid response to TKI monotherapy (Fig. 3B), suggesting that the minimal fold change of crizotinib by ALK-S1206 mutations [10, 25] in preclinical systems (Fig. 3A) is insufficient to offset the inhibition by initial achievable intratumor levels of crizotinib at its recommended starting dose [6, 22]. The presence of different *de novo ALK* kinase mutations (such as *ALK*-L1196M or G1202R that *in vitro* lead to many fold levels of resistance to crizotinib [10, 23]) could have led to a scenario where crizotinib at 250mg twice daily would have been incapable of overcoming the inhibitory hurdles of these mutants in *ALK* rearranged NSCLC and another ALK TKI should have been selected to best match the sensitivity pattern of the compound EML4-ALK plus ALK kinase domain mutant. Additional reports of these rare cases of *ALK* rearrangements co-occurring with *ALK* kinase mutations prior to starting an ALK TKI may shed light in these intriguing clinical decisions that can further improve precision oncology choices for this important cohort of NSCLCs and help explain hitherto unexplained cases of primary insensitivity to crizotinib in clinical practice.

In summary, our institutional database combined with prior cohorts demonstrates that *ALK* kinase domain mutations seem to be uncommon events in ALK inhibitor-naïve *ALK* rearranged lung adenocarcinomas but their effect on intrinsic resistance to achievable doses of clinically-available ALK inhibitors should be better evaluated in preclinical models and clinical cases.

Acknowledgments

This work was funded in part through an American Cancer Society grant RSG 11-186 (DBC), and National Cancer Institute grants P50CA090578 (DBC), R01CA169259 (SSK) and R21CA17830 (SSK).

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HIGHLIGHTS

- *ALK* rearranged lung adenocarcinomas are responsive to crizotinib
- However, *ALK* kinase mutations in crizotinib-naïve tumors have not been widely reported
- *ALK* kinase mutations were uncommon (<3%) in ALK inhibitor-naïve lung adenocarcinomas

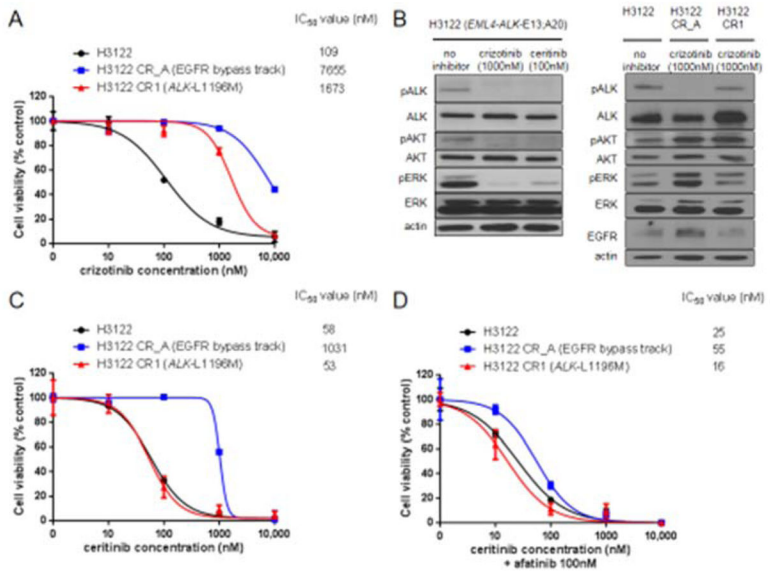
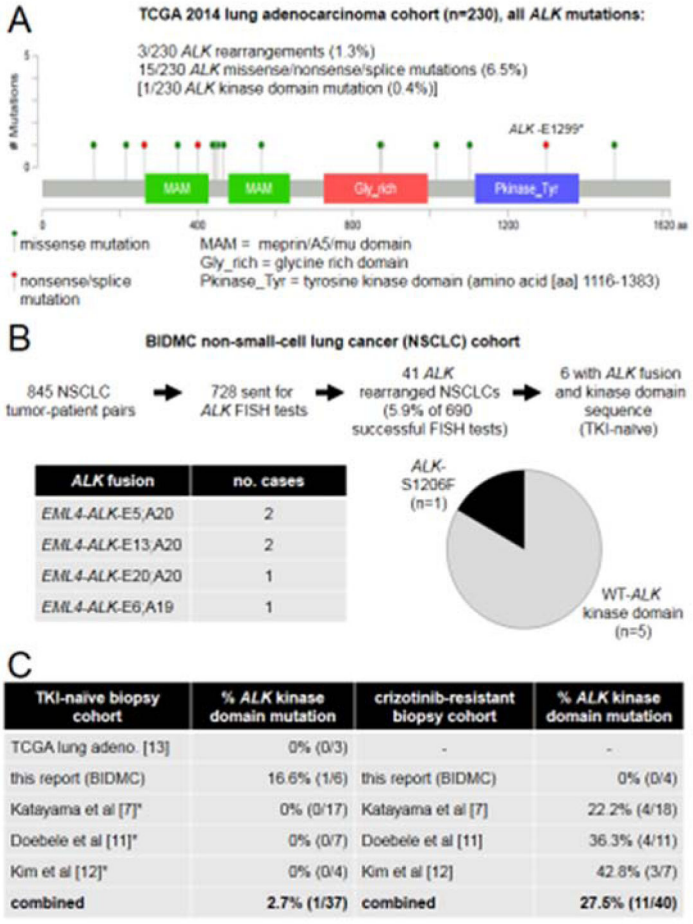


Figure 1. Preclinical models of acquired resistance to crizotinib in *ALK* rearranged lung cancers

A. Dose-inhibition curves for crizotinib using H3122, H3122 CR_A and H3122 CR1, with 50% inhibitory concentration (IC_{50}) using nanomolar (nM) concentrations indicated. B. Western blot results showing the intracellular signaling effects of crizotinib 1000nM and certinib 100nM after 6 hours of exposure to H3122 cells, with inhibition of phosphorylated (p) levels of each protein indicating drug activity. The same intracellular signaling is shown for H3122 CR_A and H3122 CR1 cells grown in the presence of continuous 1000nM of crizotinib. C. Dose-inhibition curves for ceritinib using H3122, H3122 CR_A and H3122 CR1, with IC_{50} concentrations indicated. D. Dose-inhibition curves for ceritinib in the presence of afatinib 100nM using H3122, H3122 CR_A and H3122 CR1, with IC_{50} concentrations indicated.



C

TKI-naive biopsy cohort	% *ALK* kinase domain mutation	crizotinib-resistant biopsy cohort	% *ALK* kinase domain mutation
TCGA lung adeno. [13]	0% (0/3)	-	-
this report (BIDMC)	16.6% (1/6)	this report (BIDMC)	0% (0/4)
Katayama et al [7]*	0% (0/17)	Katayama et al [7]	22.2% (4/18)
Doebele et al [11]*	0% (0/7)	Doebele et al [11]	36.3% (4/11)
Kim et al [12]*	0% (0/4)	Kim et al [12]	42.8% (3/7)
combined	**2.7% (1/37)**	**combined**	**27.5% (11/40)**

Figure 2. *ALK* kinase domain mutations in different NSCLC cohorts

A. Frequencies of *ALK* genomic changes and graphic representation of the ALK protein with *ALK* mutations identified in the TCGA 2014 lung adenocarcinoma cohort indicated. B. *ALK* rearrangements and *ALK* kinase domain mutations identified in the BIDMC NSCLC tumor-pair cohort. C. Tabulated *ALK* rearranged NSCLC cohort and percentage (%) of *ALK* kinase domain mutations in TKI-naïve and crizotinib-resistant biopsies. References to the original publication of the cohort are indicated in parenthesis. * We assumed that crizotinib-resistant samples with wild-type (WT) *ALK* kinase domain would also have lacked a mutation in the TKI-naïve setting and we excluded cases that had *ALK* kinase mutations in the crizotinib-resistant setting but were not analyzed in the TKI-naïve biopsy.

A

ALK fusion plus kinase domain mutation (Ba/F3 cells)	crizotinib IC_{50} fold change from EML4-ALK (adapted from Friboulet et al [10])	crizotinib 250mg twice daily (256ng/mL plasma [22])
EML4-ALK-E13;A20	1.0	responsive
+ ALK-S1206Y	7.9	?
+ ALK-G1269A	8.3	?
+ ALK-G1202R	14.0	resistant
+ ALK-L1196M	20.4	resistant
+ ALK-1151Tins	36	resistant

B

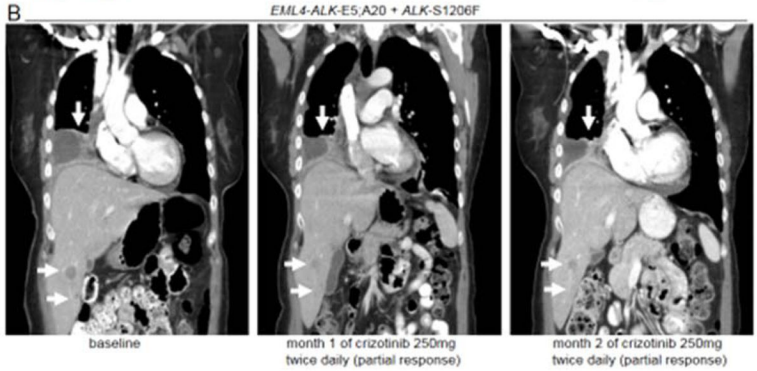


Figure 3. ALK kinase domain mutants and EML4-ALK+ALK-S1206F

A. Crizotinib 50% inhibitory concentration (IC_{50}) fold changes induced by different ALK kinase mutants in the background of EML4-ALK-E13;A20, as reported by Friboulet et al (reference 10), tabulated with possible clinical response to achievable concentrations of crizotinib 250mg twice daily. B. Computed tomography (CT) to exemplify response to crizotinib in the patient whose lung adenocarcinoma harbored *de novo EML4-ALK*-E5;A20+*ALK*-S1206F. Shown are pre-crizotinib (baseline), month 1 and month 2 of crizotinib 250mg twice daily representative images. The white arrows highlight sites of tumor burden in the thorax and liver.