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Mechanisms of Resistance to Crizotinib in Patients with *ALK* Gene Rearranged Non-Small Cell Lung Cancer

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Abstract

Purpose—Patients with anaplastic lymphoma kinase (*ALK*) gene rearrangements often manifest dramatic responses to crizotinib, a small molecule ALK inhibitor. Unfortunately, not every patient responds and acquired drug resistance inevitably develops in those that do respond. This study aimed to define molecular mechanisms of resistance to crizotinib in ALK+ non-small cell lung cancer (NSCLC) patients.

Experimental Design—We analyzed tissue obtained from 14 ALK+ NSCLC patients demonstrating evidence of radiologic progression while on crizotinib in order to define mechanisms of intrinsic and acquired resistance to crizotinib.

Results—Eleven patients had material evaluable for molecular analysis. Four patients (36%) developed secondary mutations in the tyrosine kinase domain of *ALK*. A novel mutation in the *ALK* kinase domain, encoding a G1269A amino acid substitution that confers resistance to crizotinib *in vitro*, was identified in two of these cases. Two patients, one with a resistance mutation, exhibited new onset *ALK* copy number gain (CNG). One patient demonstrated outgrowth of *EGFR* mutant NSCLC without evidence of a persistent *ALK* gene rearrangement.

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Two patients exhibited a *KRAS* mutation, one of which occurred without evidence of a persisting *ALK* gene rearrangement. One patient demonstrated the emergence of an *ALK* gene fusion negative tumor compared to the baseline sample, but with no identifiable alternate driver. Two patients retained *ALK* positivity with no identifiable resistance mechanism.

Conclusions—Crizotinib resistance in ALK+ NSCLC occurs through somatic kinase domain mutations, *ALK* gene fusion CNG, and emergence of separate oncogenic drivers.

Keywords

oncogene fusion; anaplastic lymphoma kinase; protein kinase inhibitors; drug resistance; non-small cell lung cancer

Introduction

Non-small cell lung cancer (NSCLC) is increasingly recognized as a heterogeneous set of diseases at the molecular level and these differences can drive therapeutic decision-making (1–3). Transforming rearrangements of the anaplastic lymphoma kinase (*ALK*) gene were initially identified in anaplastic large cell lymphoma (4). In 2007, an *ALK* gene rearrangement (*ALK*+) in which the 5' end of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene was fused to the 3' portion of *ALK* was identified in NSCLC (5).

Recently, crizotinib has gained FDA approval for the treatment of *ALK*+ NSCLC. Crizotinib approval was based in part on data from the phase I clinical trial which demonstrated an overall response rate of 57% and a probability of progression free survival at 6 months of 72% (6). Unfortunately, some *ALK*+ patients will not derive any benefit from crizotinib (intrinsic resistance), while patients who initially derive benefit later develop resistance (acquired resistance).

Several groups have explored molecular mechanisms of acquired resistance in a comparable clinical scenario involving resistance to EGFR TKIs in *EGFR* mutant NSCLC (7–9). The most common mechanism noted in these patients involves a change in the target of the drug, specifically a second *EGFR* mutation, T790M, that alters the binding kinetics of the reversible TKIs to the target molecule (10). In addition, clinical examples of second oncogenic drivers within the same cell harboring the *EGFR* mutation, notably *MET* gene amplification, bypassing the block caused by the TKI have also been reported (8, 11).

Isolated case reports have recently identified mutations in *ALK*+ NSCLC that occur following crizotinib therapy (12, 13). However, the frequencies with which these and any other mechanisms of resistance occur remain unknown. Here we report on the clinical and molecular details of 14 *ALK*+ NSCLC patients with intrinsic or acquired resistance to crizotinib.

Materials and Methods

Patients

Patients with *ALK*+ NSCLC, who were treated on a phase I study of crizotinib, were considered for re-biopsy upon progression on crizotinib therapy (6). Patients were consented for tissue banking under the University of Colorado Lung Cancer SPORE tissue banking protocol and all research tests were conducted under IRB approval. Formalin-fixed paraffin-embedded (FFPE), fresh frozen and/or tissue for the initiation of cell lines were collected from each patient as deemed safe and feasible by the interventional radiologist/ pulmonologist. Diagnostic sampling was prioritized over research sampling in each case. No sample size was pre-specified and the analysis performed was descriptive.

ALK molecular testing

ALK positivity was ascertained by FISH using *ALK* break-apart probes (Vysis LSI *ALK* (2p23) Dual Color, Break Apart Rearrangement Probe; Abbott Molecular). The FISH assays and analyses were performed as described previously with minor modifications (14). Using the *ALK* beak-apart probe, 3’ (red) and 5’ (green) signals physically separated by ≥2 signal diameters were considered split. Specimens were considered positive for *ALK* rearrangement if <15% of tumor cells showed split signals or single red signals. Copy number of *ALK* rearrangement was based upon determination of the mean of split and isolated red signals per tumor cell (15). At least 100 tumor cells were analyzed per specimen.

RNA from either FFPE or Frozen tissue was processed using the RecoverAll™ Total Nucleic Acid Isolation Kit from Ambion (Austin, TX). RT-PCR was carried out using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase from Invitrogen (Carlsbad, CA) with primers to *EML4-ALK*. PCR products were either resolved on an agarose gel or analyzed with a Bioanalyzer from Agilent Technology (Santa Clara, CA), excised, and purified using Wizard SV Gel and PCR Clean Up Kit from Promega (Madison, WI) then sequenced as described below. Primers used for RT-PCR or multiplexed RT-PCR of the *EML4-ALK* gene fusion transcript are listed in Supplementary Table 1 or have been previously described (16, 17).

EGFR, KRAS, ALK and Short Tandem Repeat Genotyping

Genomic DNA was isolated from manually microdissected FFPE tumor samples using the QiaAmp FFPE DNA isolation kit from Qiagen (Valencia, CA). Samples were PCR amplified using custom primer sets for exon 2 of *KRAS*, exons 19 through 21 of *EGFR*, or exons 21–25 of *ALK* (see Supplementary Table 1 for primers) and directly sequenced using the ABI Big Dye Thermocycle Sequencing kit and analyzed on an ABI 3730 DNA Sequencer (16). Mutation analysis was assisted by the use of Mutation Surveyor software v3.97–4.0.0 from SoftGenetics (State College, PA). The reference sequence for *ALK* sequence comparison used was NM_004304.4, for *EML4* NM_019063.3, for *EGFR* NM_005228.3, and for *KRAS* NM_004985.3. We have used the colloquial single letter amino acid substitution for mutations described here rather than the official nomenclature for simplicity (e.g., L1196M rather than p.Leu1196Met).

The SNaPshot assay for evaluation of multiple oncogenic mutations in *APC, AKT1, BRAF, CTNNB1, EGFR, FLT3, JAK2, KIT, KRAS, MAP2K1* (*MEK1*), *NOTCH1, NRAS, PIK3CA, PTEN*, and *TP53* was performed by amplification using 13 multiplexed PCR reactions followed by single nucleotide base extension reactions. The products were separated by capillary electrophoresis and analyzed using GeneMapper 4.0 as has been previously described (18). To confirm patient identity across multiple samples in select cases, short tandem repeat analysis was performed on extracted genomic DNA using the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Carlsbad, CA).

Cell lines and Reagents

Primary cell lines were derived from patients by placing fresh tumor tissue into sterile tissue culture dishes and culturing in RPMI supplemented with 10% FBS. A new cell line, CUTO-1 (Colorado University Thoracic Oncology), derived from a tumor biopsy sample of patient #10 is described here. Ba/F3 cells (kindly provided by James DeGregori) were cultured in RPMI supplemented with 10% FBS and 1ng/ml of IL-3 from R&D Systems (Minneapolis, MN). 293T human embryonic kidney cells and NIH3T3 mouse fibroblast cells were obtained from ATCC (Manassas, VA) and grown in DMEM supplemented with 5% FBS. NCI-H3122 and NCI-H2228 (kindly provided by John D. Minna and Adi F. Gazdar, UTSW) were grown in RPMI with 10% FBS.

Mouse monoclonal against total ALK (#3791), rabbit polyclonal against phosphorylated ALK Tyr 1278/1282/1283 (#3983), mouse monoclonal against AKT (#2920), mouse monoclonal against total ERK p42/44 (#9107), rabbit polyclonal against phosphorylated ERK Thr202/Tyr204 (#9101), mouse monoclonal against total STAT3 (#9139), rabbit polyclonal against phosphorylated STAT3 Tyr705 (#9145) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against phosphorylated AKT Ser474 (sc-135651) and mouse monoclonal antibodies against α-tubulin (#sc-8035) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye® 680LT- and 800CW-conjugated goat anti-mouse secondary antibodies were purchased from LI-COR Biotechnology (Lincoln, NE). PF-02341066 (crizotinib) was kindly provided by Pfizer and dissolved in DMSO for experiments.

Lentiviral constructs and transduction

The *EML4-ALK* gene fusion containing *EML4* exons 1–6 variant and *ALK* exons 20–29 (E6a;A20) was cloned by RT-PCR from mRNA isolated from the cell line, H2228, and inserted into the lentiviral expression plasmid, pCDH-MCS1-EF1-puromycin from Systems Biosciences (Mountain View, CA). Point mutations were introduced into the *EML4-ALK* fusion gene using the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technology (see Supplementary Table 1 for primers). Lentiviral transduction of Ba/F3, NIH3T3 and H3122 were carried out as previously described (19).

Immunoblotting

Immunoblotting was performed as previously described with minor modifications (20). Briefly, cells were lysed in modified RIPA buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail purchased from Thermo Scientific (Waltham, MA). Total protein was separated by SDS-PAGE, transferred to nitrocellulose, and stained with the indicated primary antibodies. Protein detection was achieved by imaging with an Odyssey Imager and Odyssey Version 3.0 image analysis software from LI-COR (Lincoln, NE).

Proliferation and Soft Agar Colony Assays

Proliferation of Ba/F3 cells was measured using the Cell-titer 96 Aqueous Proliferation Assay from Promega (Madison, WI) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates 24 hours prior to drug treatment and proliferation was measured 72 hours after treatment. The absorbance at 490nm was measured in 96-well plates using a Microplate Reader from Molecular Devices (Sunnyvale, CA). The IC_{50} values were calculated using Prism v5.02 from GraphPad Software (La Jolla, CA).

In order to measure anchorage-independent growth and its inhibition by crizotinib, Ba/F3 cells expressing non-mutated or mutated cDNAs encoding *EML4-ALK* (E6a;A20) were suspended in media containing 0.4% agar and plated in 6-well plates containing media + 0.5% agar per well. Wells were fed every 3 days with media with the indicated concentrations of crizotinib and incubated for 14 days. Colonies were stained for 24 hours with NBT and photographs were taken for colony quantification with Metamorph Software from Molecular Devices (Sunnyvale, CA).

Results

Re-biopsy of patients following progression on crizotinib

Fourteen *ALK*+ patients underwent 15 re-biopsy procedures following radiologic evidence of disease progression/lack of response on crizotinib (Fig. 1B and S1A, Table S1). Typically, diagnostic biopsies were used as the pre-crizotinib (baseline) sample for comparison purposes within this study. Two patients were biopsied following disease progression at the first evaluation on crizotinib (intrinsic resistance). Twelve patients underwent biopsy following initial benefit then progression (acquired resistance) after median time on crizotinib of 8.9 months (range, 3.8–21.1 months). One patient underwent two separate biopsy procedures post-initiation of crizotinib, one after a lack of response (stable disease), followed by biopsy of a separate lesion after disease progression per RECIST (version 1.0).

Three patients (#1–3) failed to yield evaluable material from their biopsy. Frozen tumor tissue was collected from 4 patients and FFPE was collected on 11 patients. All eleven patients with evaluable tissue underwent repeat *ALK* FISH testing (Table 1). Attempts to propagate a cell line occurred for 8 patients. Currently only one cell line, from patient #10, is propagating at a rate permitting evaluation.

ALK kinase domain mutations as a resistance mechanism to crizotinib

Acquired mutations clustering around the kinase domain ATP binding site are a well-recognized mechanism of acquired resistance to tyrosine kinase inhibitors (TKIs) (7, 21, 22). We therefore performed direct sequencing of *ALK* exons 21–25, encoding the kinase domain. Four patients (#4–7) were found to have point mutations in the kinase domain of *ALK* (Table 2). None of these patients had sufficient tissue in their pre-crizotinib biopsy to determine if these mutations were detectable prior to crizotinib therapy. Patients #4 and #5 were found to have the previously described mutation encoding the L1196M substitution (data not shown). Two patients (#6 and 7) demonstrated the presence of a novel mutation that encodes a G1269A substitution (Table 2 Supplemental Fig. S2A, and data not shown).

A multiplexed RT-PCR assay for *EML4-ALK* was performed when possible to determine the variant of the *ALK* gene fusion and we successfully confirmed the presence of *EML4-ALK* in 4 patients. Interestingly, patient #7 demonstrated a new variant fusing exon 6 of *EML4* to exon 19 of *ALK* (E6;A19) (Supplemental Fig. S3, Table 2). An *EML4-ALK* specific RT-PCR was performed on mRNA in order to selectively amplify and sequence the expressed transcripts of *EML4-ALK* (Supplemental Fig. S3A).

The amino acid substitutions encoded by the observed mutations were mapped onto the crystal structure of the ALK kinase domain (Fig. 1A and B) (23). All of the mutations detected in this series were found in, or near, the long narrow groove that comprises the binding pocket for both ATP and crizotinib. The L1196M substitution has been previously described in a patient with crizotinib resistance and is homologous to the gatekeeper mutations identified in *BCR-ABL* (T315I) and *EGFR* (T790M) (7, 12, 21).

The Gly 1269 residue is positioned at the end of the narrow ATP-binding pocket of ALK (Fig. 1A and B). Substitution of Gly1269 with the larger Ala residue would be expected to reduce binding of crizotinib due to steric hindrance. To determine whether the G1269A substitution produced resistance to crizotinib, the mutation encoding this substitution was generated in a cDNA encoding the E6a;A20 variant of *EML4-ALK*. Lentiviral vectors encoding wild-type *EML4-ALK* (E6a;E20) or the same cDNA encoding G1269A, C1156Y, or L1196M substitutions or empty vector were introduced into Ba/F3 cells. Proliferation assays in the presence of increasing doses of crizotinib were performed (Fig. 1C). The G1269A mutation induced crizotinib resistance that was intermediate between the previously identified mutations, C1156Y and L1196M. Similar results were obtained when these constructs were introduced into the NIH3T3 cell line and colony formation was measured in soft agar (Supplemental Fig. S4). Consistent with the observed cell line resistance, ALK phosphorylation and phosphorylation of downstream effectors, ERK, STAT3, and AKT were preserved at higher doses of crizotinib in the G1269A mutant compared to wild-type *EML4-ALK* (Fig. 1D and E).

Preclinical and clinical evidence suggests that the gatekeeper mutation T790M in *EGFR* mutant NSCLC may provide a growth disadvantage compared to NSCLC with an *EGFR* mutation lacking T790M (24, 25). We therefore examined the relative fitness of the G1269A, C1156Y, and L1196M mutations compared to wild-type *EML4-ALK* (variant E6a;A20) in the Ba/F3 cell system (Fig. 1F). We did not detect a growth disadvantage for any of the resistant mutations compared to wild-type. Indeed, the *EML4-ALK* constructs harboring resistance mutations induced increased proliferation compared to non-mutated *EML4-ALK* in the absence of crizotinib.

Copy number gain of the *ALK* gene rearrangement as a mechanism of crizotinib resistance

A gain in *ALK* gene fusion copy number has recently been implicated as a mechanism of resistance to crizotinib *in vitro* (26). In addition to standard FISH analysis for *ALK*, we measured the number of copies per cell of the *ALK* gene rearrangement before and after crizotinib treatment (15). Copy number gain (CNG) was defined as more than two-fold increase in the mean of the rearranged gene per cell in the post-treatment specimen compared with the pre-treatment specimen. Two patients demonstrated a marked increase in abnormal signal copy number (#7 at 5-fold and #8 at >4 fold), consistent with CNG of the *ALK* gene fusion (Table 1Fig. 2A and B). The CNG in these two patients was due both to more copies of the *ALK* rearrangement per cell and more cells displaying the rearrangement pattern.

EGFR mutations as a mechanism of crizotinib resistance

Patient #9 underwent two separate biopsy procedures each of different lesions. The first was performed after 61 days on crizotinib after the initial re-staging scans demonstrated stable disease, which was considered unusual given the responses seen in the majority of ALK positive patients treated with crizotinib (6). This biopsy demonstrated a lack of an *ALK* gene rearrangement by FISH (Fig. 2C). Further evaluation by direct sequencing demonstrated the presence of an *EGFR* exon 21 mutation encoding the L858R substitution (Table 2 Supplemental Fig. S2B) that was not present in the initial transbronchial biopsy used to establish the *ALK*+ diagnosis (data not shown). The *EGFR* mutation on this specimen was confirmed by SNaPshot analysis (data not shown). Interestingly, a second biopsy performed on a progressing liver lesion after 113 days on crizotinib did show an *ALK* gene rearrangement, but no evidence of an *ALK* kinase domain mutation, an *EGFR* mutation or of any other abnormal oncogenes as assessed by SNapSHOT (Tables 1 and 2, data not shown). To confirm that all samples were indeed from the same patient, the original diagnostic biopsy and both re-biopsy samples were fingerprinted using STR analysis, confirming the common genetic origin of the samples (data not shown).

Of note, Patient #1, who underwent a post-crizotinib biopsy but had no evaluable material (Supplemental Table S1), demonstrated the presence of an *EGFR* exon 20 mutation (S768I) in the pre-crizotinib biopsy sample in addition to the presence of an *ALK* gene rearrangement by FISH analysis. This patient received erlotinib therapy for more than 9 months before discontinuing it and beginning crizotinib.

KRAS mutations as a mechanism for crizotinib resistance

Patient #10 received only 27 days of crizotinib before disease progression was evident (Supplemental Table S1). Testing of the re-biopsy sample showed a persistent *ALK*+ FISH test but no further molecular testing was performed as the remainder of the tumor sample was used to initiate a cell line (Supplementary Fig. S5). Initial analysis of this cell line demonstrated marked resistance to crizotinib in comparison to two known *EML4-ALK* positive NSCLC cell lines (Fig. 3A). FISH analysis of this cell line later demonstrated no evidence of an *ALK* gene rearrangement and repeated RT-PCR analysis failed to show evidence of an *EML4-ALK* gene transcript (data not shown). SNaPshot analysis of the cell line demonstrated the presence of a *KRAS* G12C mutation and this was confirmed by direct sequencing (Supplemental Fig. S3C). Given the short duration until progression we asked whether this mutation was detectable in the pre-crizotinib sample. Direct sequencing of the microdissected pre-crizotinib biopsy, which had not been previously analyzed for *KRAS*, demonstrated the presence of the *KRAS* G12C mutation (Supplemental Fig. S3C).

Patient #11 experienced a partial response to treatment, before progression in the liver after 7 months, when a biopsy was performed and demonstrated persistence of an *ALK* gene rearrangement as well as a *KRAS* mutation encoding the G12V substitution (Tables 1 and 2, Supplemental Fig. S3D). Analysis of the diagnostic biopsy showed no evidence of a pre-existing mutation in *EGFR* or *KRAS* (Supplemental Table S1).

In patient #11, we were unable to determine whether the *ALK* gene rearrangement and *KRAS* mutation occurred in the same or different tumor cells. Whereas in the case of patient #10, given that an *ALK* negative, *KRAS* positive cell line could be generated from a lesion in whom both abnormalities were present in the biopsy this means that *ALK* and *KRAS* positive cells must have existed as separate subclones within the same tumor. To further explore whether acquisition of a *KRAS* mutation could serve as a direct mechanism of acquired resistance to crizotinib in *ALK* positive cells, we asked whether expression of a mutant *KRAS* G12V could elicit crizotinib resistance in an *EML4-ALK* positive cell line (H3122), which is normally sensitive to crizotinib. *KRAS* G12V was selected because it was the form observed in patient #11, where the co-existence in the same cells could not be formally evaluated. Mutant *KRAS* G12V or empty vector was introduced into H3122 and cell proliferation was measured after exposure to increasing doses of crizotinib (Fig. 3B). The IC_{50} of H3122 expressing *KRAS* G12V was not significantly different from H3122 harboring the empty vector.

Emergence of an ALK gene fusion negative tumor

All post-crizotinib biopsy samples with evaluable tumor tissue underwent repeat *ALK* FISH testing. In patient #10, an *ALK* gene fusion was not observed in the cells which were propagated from the rebiopsied supraclavicular fossa lesion, which were later shown to be *KRAS* mutant. In patient #9, an *ALK* gene fusion was not observed in the rebiopsied supraclavicular fossa lesion, which was later shown to harbor and *EGFR* mutation. Patient #12 also lacked an *ALK* gene fusion as detected by FISH in the rebiopsy of the infraclavicular lymph node (Table 1Fig. 2D). RT-PCR of the post-crizotinib sample from patient #12 using a multiplexed assay to detect different *EML4-ALK* variants also failed to demonstrate the presence of an *ALK* gene fusion (data not shown). Unlike with patients #10 and #11, no other abnormality was detected in the evaluated genes in this patient using the SNaPshot assay.

Unknown mechanisms of crizotinib resistance

Patients #13 and #14 demonstrated the presence of an *ALK* gene rearrangement by FISH analysis following progression on crizotinib (Table 1). Patient #13 showed no evidence of *ALK* gene CNG or loss and no evidence of an *ALK* kinase domain mutation. Patient #14 showed no evidence of *ALK* gene CNG or loss, no evidence of an *ALK* kinase domain mutation, and no evidence of an *EGFR* or *KRAS* mutation (Table 1 and 2).

Discussion

Here we describe the molecular mechanisms of resistance in a large series of *ALK*+ NSCLC patients with progression o n crizotinib therapy. Previous studies have described the development of *in vitro* resistance mechanism using cell lines grown in the presence of crizotinib (26–29). Selected clinical cases of crizotinib resistance have been described, but without a denominator to estimate the frequency of the given abnormalities (12, 13). In this study, we successfully obtained molecular data on 11 of 14 rebiopsied *ALK*+ NSCLC patients with progression on crizotinib. A specific potential resistance mechanism was identified in 9 of these cases (Fig. 4A).

Precedent exists for the emergence of kinase domain mutations as mechanisms of resistance, most notably T315I in *BCR-ABL* and T790M in *EGFR* (7, 21, 22). We identified four patients (36%) with acquired resistance mutations in *ALK*. Two patients had the previously described L1196M mutation, in the classical gatekeeper position homologous to T315I and T790M (12). L1196M levels were low in one patient but were confirmed by RFLP analysis (data not shown). Two patients demonstrated a novel G1269A mutation. One of these patients also harbored a novel *ALK* gene fusion involving exon 6 of *EML4* and exon 19 of *ALK* (Supplementary Fig. 3). All previously published *ALK* gene fusions involve exon 20 of *ALK*. Mutations at position G1269 have previously been identified using an *in vitro* mutagenesis screen (29). *In vitro* studies with G1269A demonstrate persistent ALK phosphorylation and downstream effector phosphorylation at higher doses of crizotinib than in the wild-type. Decreased growth inhibition from crizotinib was also observed with G1269A relative to the wild-type. Interrogation of the ALK crystal structure bound to crizotinib reveals G1269 to be critically situated in the ATP-binding pocket (Fig. 1A and B). Crizotinib binding in this pocket is unlikely to be able to tolerate larger amino acid substitutions in either G1269 or L1196. Replacement of the Leu side chain with a longer thioether side chain (L1196M) has been shown to significantly compromise the interaction with crizotinib (12). Similarly, a bulkier Ala in place of Gly1269 would preclude proper binding of the halogenated aromatic ring of crizotinib (Fig. 1A and B). One of the patients with a G1269A mutation also demonstrated a novel *EML4-ALK* fusion variant (E6; A19). All previously described gene fusions involving *ALK* in NSCLC fuse different exons of *EML4* to exon 20 of *ALK*.

The observation that 4 of 10 resistant samples had *ALK* kinase domain mutation suggests that resistance mutations in *ALK*+ NSCLC will likely be a common mechanism of resistance to crizotinib, similar to that of EGFR TKI resistance (9). However, unlike EGFR TKI resistance in NSCLC, there appears to be a greater diversity of resistance mutations as 4 different mutations have now been identified in *ALK*+ NSCLC patients (12, 13). This is reminiscent of CML patients treated with imatinib, in which multiple different mutations in the ABL kinase domain have been identified (30). One can speculate that the kinase domain of EGFR may be more constrained given the presence of an existing activating mutation and may only be able to tolerate a very limited set of mutations that inhibit drug binding but still allow constitutive activation. In contrast EML4-ALK, like *BCR-ABL*, has a native kinase domain structure that may be able to tolerate a wider spectrum of resistance mutations that inhibit crizotinib binding but still allow constitutive activation. Consistent with this hypothesis is the *in vitro* data demonstrating that the *ALK* resistance mutations studied here exhibited enhanced growth compared to the non-mutated *EML4-ALK* in an isogenic cell line. In contrast, *EGFR* T790M mutations confer impaired growth compared to cells carrying *EGFR* activating mutations alone (25).

The existence of multiple different resistance mutations in *ALK* may have important clinical implications. First, testing for resistance mutations will require direct sequencing of multiple exons or a multiplexed assay looking for different specific mutations as data emerge. Second, the possibility of different mutations existing in the same patient has to be considered, increasing the difficulty of detecting each mutation. This is in addition to multiple other mechanisms that could affect detection including allelic dilution or a mixture of mutant and non-mutant cells in the biopsy sample (31, 32). Consequently, some resistance mutations may be missed and could conceivably be present in either of the patients in our study who retained *ALK* but appeared to have an unknown mechanism of resistance, or in the patient with CNG alone as their apparent mechanism of resistance. CNG alone has been described as a potential mechanism of resistance *in vitro*, however this appeared to be a precursor state to the development of a resistance mutation (26). Finally, based on our own and others preclinical work different mutations appear to have different sensitivity rank orderings to crizotinib and this ordering may differ between ALK inhibitors (Fig. 1C and Supplemental Fig. S4) (29, 33). Therefore, the optimal exposure of any ALK inhibitor in the TKI naïve setting may differ from that in the acquired resistance setting. In addition, beyond dosing and toxicity issues, different ALK inhibitors may have to be prioritized depending on the specific mutation involved.

Seven out of 11 patients in this study did not have *ALK* kinase domain mutations, which led us to investigate alternate oncogenes as contributors to resistance. Prior studies have demonstrated the coexistence of both an *EGFR* activating mutation or a *KRAS* mutation and an *ALK* gene rearrangement in the same tumor sample (13, 34–36). Here we describe 2 cases with a *KR*AS mutation (one of which was retrospectively detected in the pre-crizotinib specimen) and one patient with an *EGFR* activating mutation at the time of resistance. An additional patient had a coexistent *EGFR* S768I mutation and an *ALK* gene rearrangement in the pre-crizotinib tumor sample, but no evaluable tissue following progression on crizotinib. The presence of *EGFR* and *ALK*, or *KRAS* and *ALK* in two of the pre-crizotinib tumor samples is consistent with results from a large cohort of *ALK*+ patients showing that 3 of 38 (8%) *ALK*+ NSCLC patients also demonstrated the presence of a KRAS or EGFR mutation (37).

The presence of multiple oncogenes in a tumor sample raises the question of whether these occur in the same tumor cells or different tumor cells and how these different oncogenic drivers arise (Fig. 4B). In *EGFR* mutant NSCLC with acquired resistance to EGFR TKIs via *MET* gene amplification, *in vitro* data suggest that *MET* gene amplification occurs as a secondary event in the same tumor cell as depicted in model #1 (Acquisition of Second Oncogenic Driver) (11). This can be detected at low levels in the biopsies taken prior to EGFR TKI treatment (38). Treatment with an EGFR TKI selects for those clones with an *EGFR* activating mutation and *MET* amplification. We suggest a different model by which an alternate oncogene provides resistance to crizotinib in *ALK*+ NSCLC. In model #2

(Emergence of a Separate Oncogenic Driver), *EGFR* or *KRAS* mutations exist in separate subclonal populations that lack an *ALK* gene rearrangement. The presence of *EGFR/ALK* or *KRAS/ALK* double positive results in biopsies prior to crizotinib treatment cannot distinguish model #1 from model #2. However, the outgrowth of a *KRAS* mutant, *ALK* negative cell line from patient #10 confirms that model #2 can occur clinically. Similarly, although we did not have cell line data from patient #11 who manifested both *ALK* and *KRAS* positivity following progression, the fact that the introduction of the *KRAS* mutation seen in patient #11 into an *ALK* positive cell line did not appear to alter their sensitivity to crizotinib *in vitro* argues against model #1, at least with regard to *KRAS* and *ALK*. In contrast to *KRAS* activating mutations, introduction of an *EGFR* activating mutation into H3122 cells *in vitro* is sufficient to induce crizotinib resistance, suggesting that in some situations the acquisition of a second driver within the same ALK positive cells could act as a mechanism of resistance clinically (model #1) (13). However, as an *EGFR* activating mutation was noted in progressing lesions without evidence of a persistent *ALK* gene rearrangement in patient #9, clinically, even for *EGFR*, model #2 can also exist. We also cannot exclude the presence of two separate primary cancers in this patient. Formally, whether separate oncogenic driver subclones arise completely independently, whether they share a common progenitor that lacks either driver and these drivers are developed independently as later events, or whether both drivers co-exist within the same cell, and then one or other is lost in subclonal evolution is unclear.

Emergence of an *ALK* gene fusion negative tumor was observed in one patient where another oncogenic driver was not identified. Given the limited tumor sample and lack of a cell line in this patient, we were unable to query the presence of other oncogenic drivers beyond selected alleles of the genes evaluated in the SNaPshot panel. However, given that the emergence of an *ALK* negative tumor was associated with definite evidence of a separate oncogenic driver in both patients #9 and #10, the assumption is that some other as yet unidentified oncogenic driver is present in these cells for them to persist. It should also be noted that in cases of the emergence of an *ALK* FISH negative tumor that the percent positive cells was not zero, consistent with background noise in the break apart FISH assay as described previously (39).

ALK CNG was observed in two patients. One patient had *ALK* CNG in conjunction with an *ALK* mutation and one had CNG without another detectable oncogene or mutation being present. The percentage cells positive for a rearrangement was greater following CNG consistent with previous findings (15). A cell line that was partially crizotinib resistant *in vitro* apparently due to *ALK* gene fusion amplification alone was recently described (26). Increase of *BCR-ABL* copy number in CML serves as a precedent for this mechanism of resistance (40). In contrast, *EGFR* CNG has been more associated with EGFR TKI sensitivity rather than resistance (41, 42).

We have described a series of *ALK* positive NSCLC patients with intrinsic or acquired resistance to crizotinib. Multiple different mechanisms appear to occur (Fig. 4). We would predict that in patients in whom *ALK* remains the dominant driver of their cancers (those with kinase domain mutations and *ALK* CNG), different therapies primarily directed towards the ALK protein, either TKIs or HSP90 inhibitors, may be beneficial (43, 44).

However, when separate or second drivers occur – drug combinations or broader based treatments such as cytotoxic chemotherapies may be required (13, 45). If understanding the heterogeneity present in NSCLC is important enough to direct patients to the correct initial therapy then it is becoming clear that re-biopsying and re-analyzing cancers as they stop responding may be equally important as resistance is not occurring through a single mechanism (37). Fully understanding the basis and frequency of the different mechanisms of resistance to crizotinib that are emerging will help us to continue to exploit personalized medicine approaches when considering how to overcome crizotinib resistance in *ALK*+ NSCLC patients in the future.

Translational Relevance

Crizotinib is an orally bioavailable, small molecule tyrosine kinase inhibitor (TKI) that was approved by the FDA in 2011 for use in patients with *ALK* FISH+ NSCLC. Crizotinib provides significant clinical benefit for ALK positive NSCLC. Unfortunately, it is expected that not all ALK+ patients will benefit and those patients who do respond will eventually experience resistance of their NSCLC to crizotinib. In the same way that using targeted therapies in patients with molecularly defined cancer has been successful, it is anticipated that understanding the molecular mechanisms of resistance to targeted therapies in cancer will lead to therapeutic strategies to overcome resistance in the same population of patients. Here we describe the molecular mechanisms of resistance to crizotinib in a series of *ALK*+ NSCLC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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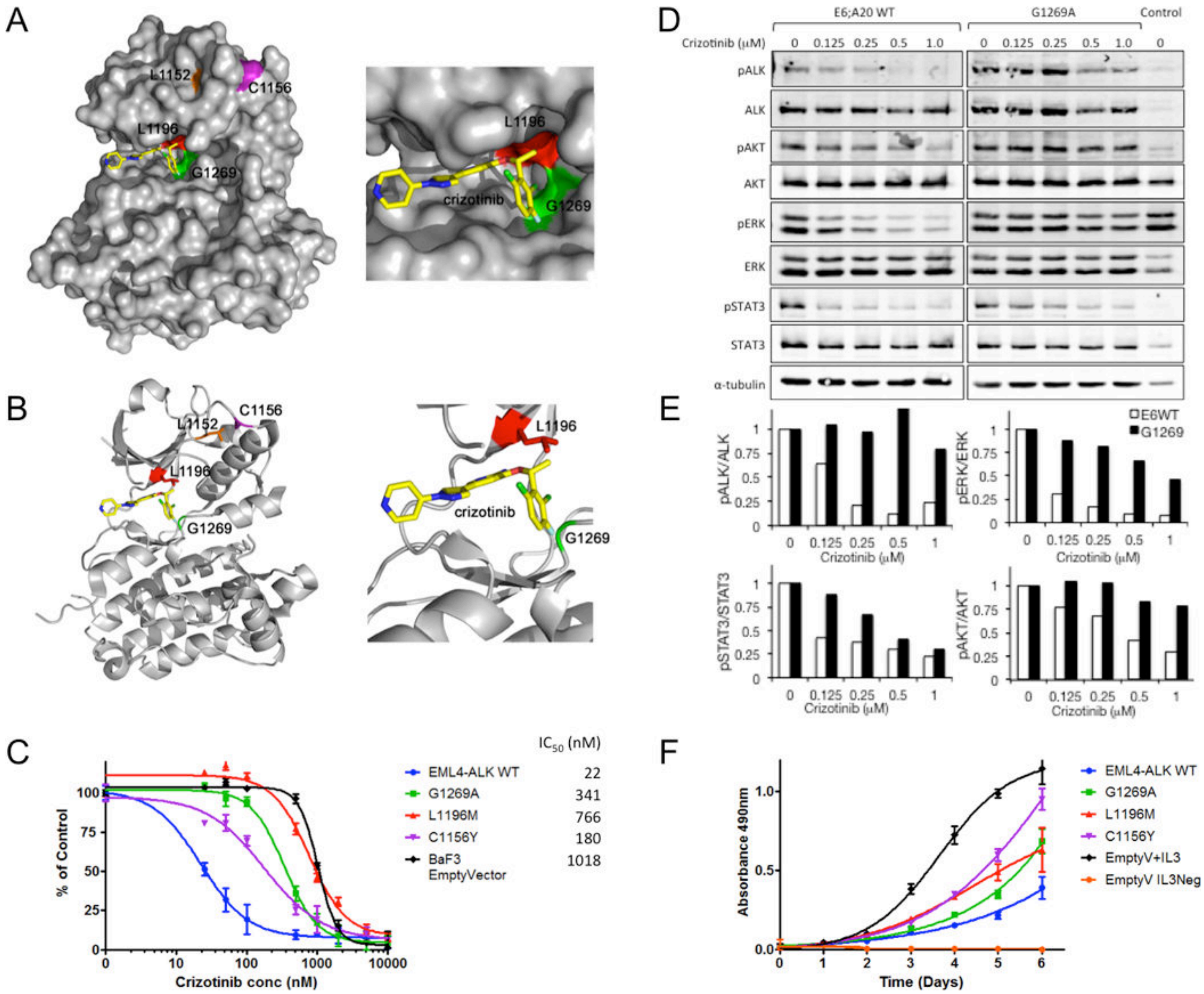

Figure 1. Identification and characterization of a novel *ALK* Kinase domain mutation in patients

Surface (A) and ribbon (B) models of the ALK kinase domain in complex with crizotinib (PDB 2XP2). Insets to the right represent a magnified view of the ATP- binding pocket where crizotinib is located. (A) and (B) were generated using the PyMol molecular graphics system. (C) Ba/F3 cells expressing wild-type *EML4-ALK* (E6;A20), or the same *EML4-ALK* construct with the specified *ALK* kinase domain mutations or empty vector were treated with the indicated concentration of crizotinib and viable cells were measured after 72 hours and then plotted relative to untreated controls. Ba/F3 cells expressing empty vector were grown in the presence of IL-3. (D) SDS-PAGE and immunoblotting analysis to detect the indicated proteins in cell lysates from NIH3T3 cells expressing wild-type *EML4-ALK* (E6;A20) and *EML4-ALK* (E6;A20) G1269A treated with the indicated doses of crizotinib for 5 hours. NIH3T3 with empty vector are also shown as a control. (E) Quantitation of western blot in (D) is graphically represented using LiCor image analysis software. (F) Ba/F3 cells expressing wild-type *EML4-ALK* (E6;A20), or the same *EML4-ALK* construct with the specified *ALK* kinase domain mutations or empty vector (with and without IL-3) were plated and viable cells were measured at the indicated time points and plotted.

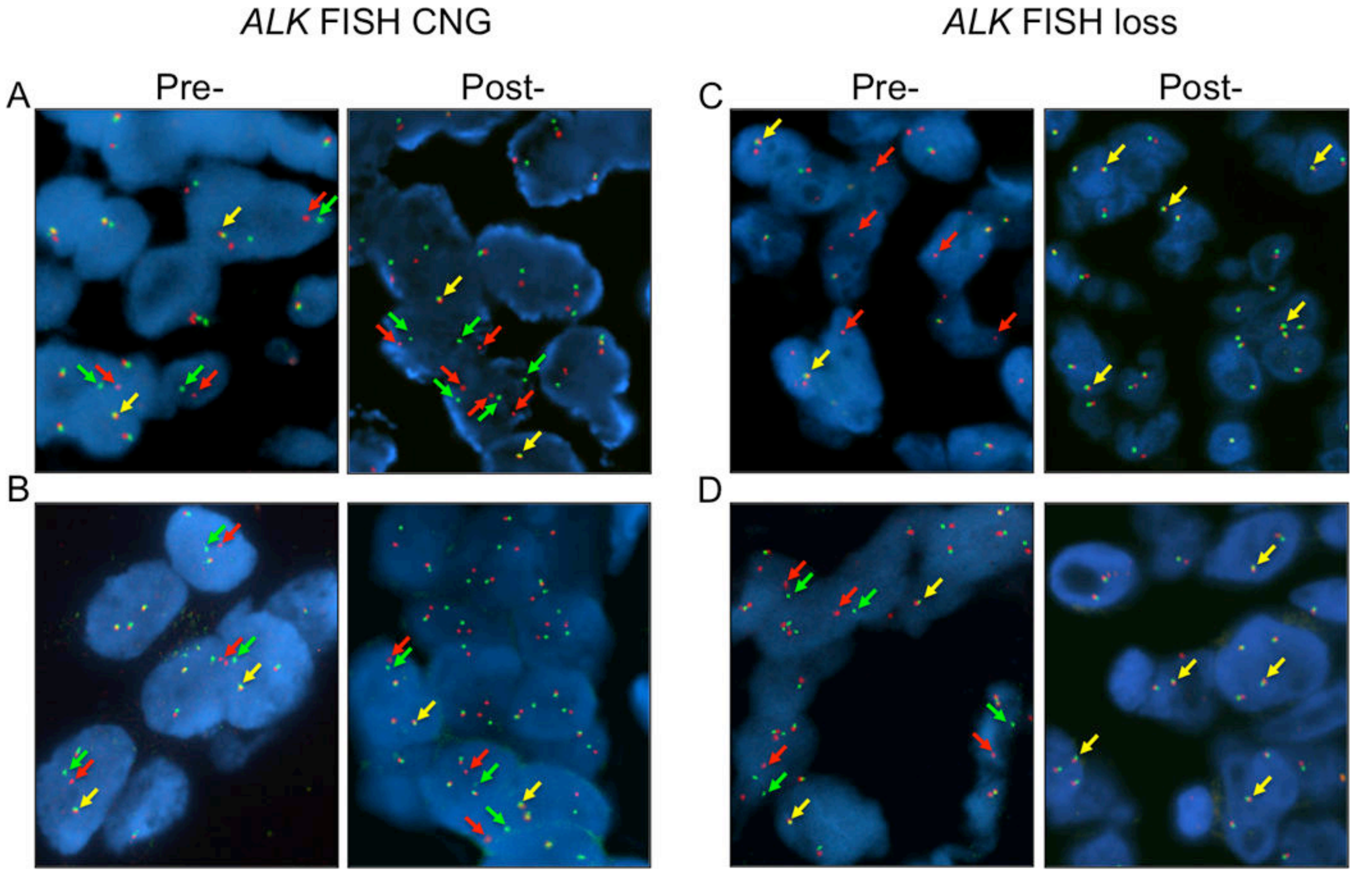

Figure 2. ALK FISH pattern changes from pre- to post-crizotinib tumor samples

FISH analysis of patients #6 (A) and #7 (B) before crizotinib treatment (left) and following progression on crizotinib (right) demonstrating a gain of split green (5') and red (3') *ALK* signals per each tumor cell. FISH analysis of patients #8a (C) and 11 (D) before crizotinib treatment (left) and following progression on crizotinib (right) demonstrating loss of split green (5') and red (3') *ALK* signals.

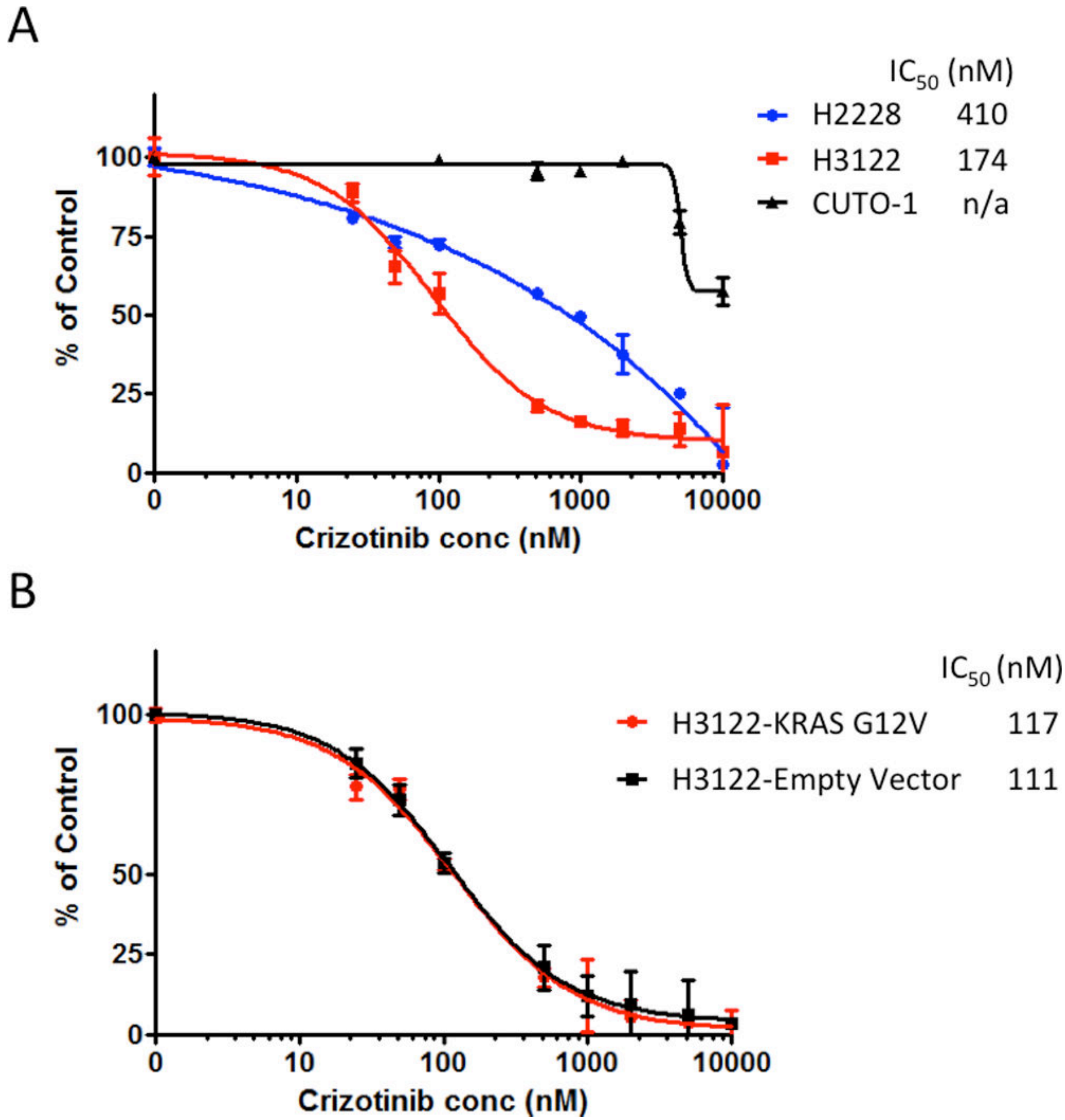

Figure 3. Alternate activating oncogenes in patients with *ALK*+ NSCLC

(A) H3122, H2228, and CUTO-1 cells (from patient #10) were treated with the indicated concentration of crizotinib and viable cells were measured after 72 hours and then plotted relative to untreated controls. (B) H3122 cells with expression of *KRAS* G12V or empty vector were treated with the indicated concentration of crizotinib and viable cells were measured after 72 hours and then plotted relative to untreated controls.

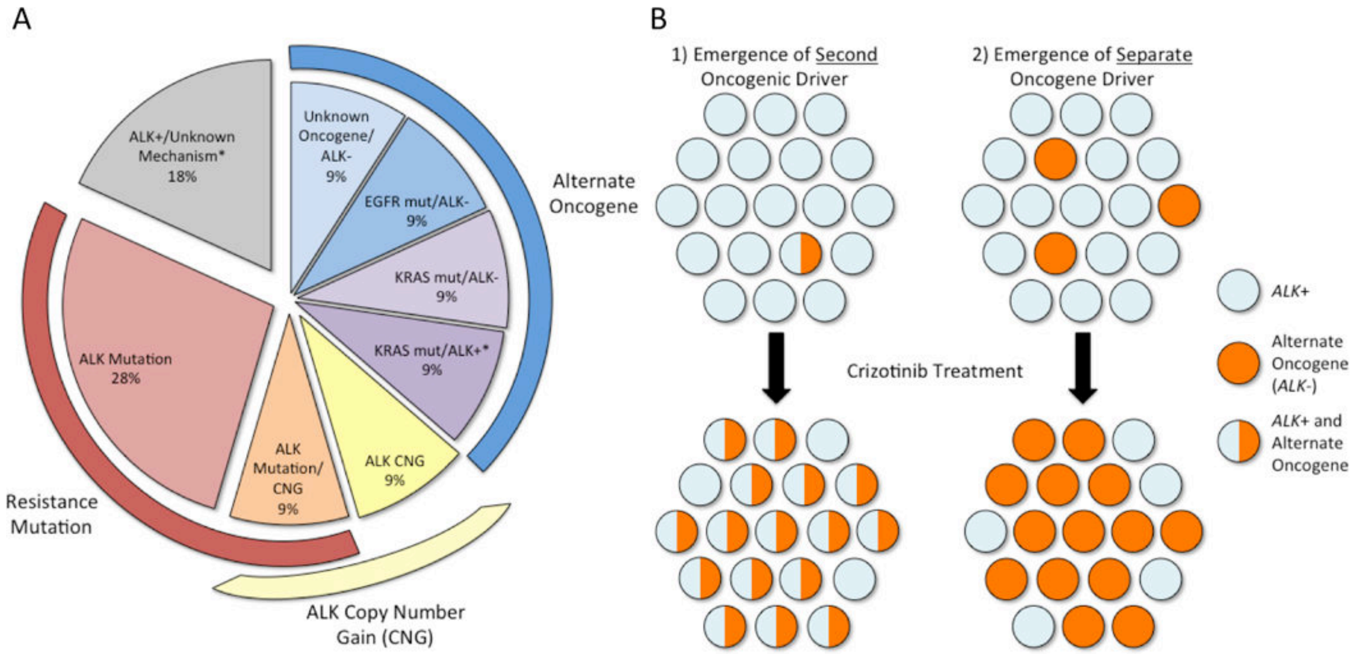

Figure 4. Relative frequencies of crizotinib resistance mechanisms in *ALK*+ NSCLC patients and models for potential mechanisms of alternate oncogene acquisition

(A) The wedges represent different molecular mechanisms of resistance identified in ALK+ NSCLC patients in this study. The blue arc represents presumed or confirmed presence of an alternate oncogene. The yellow arc represents copy number gain (CNG). The red arc represents the presence of an *ALK* kinase domain mutation. The grey wedge represents those patients where an *ALK* gene rearrangement was observed, but no mechanism of resistance was identified. *Denotes inclusion of one patient with intrinsic resistance within this category. (B) Model #1 depicts the low level presence of a second oncogenic driver in the same cell as an *ALK* gene rearrangement, which following treatment with crizotinib becomes the dominant clone. Model #2 depicts the presence of separate clonal populations, some with an *ALK* gene rearrangement as the driver and others with an alternate oncogene driver (e.g., *KRAS* or *EGFR*). Following treatment with crizotinib, the non-ALK clones become the dominant clone.

Table 1

ALK FISH comparison before and after crizotinib on evaluable patients

	Pre-crizotinib			Pre-crizotinib				
Patient #	***ALK* FISH % cells positive**	***ALK* FISH pattern[§]**	**Abnormal*ALK* copy number/cell[*]**	***ALK* FISH**	***ALK* FISH % cells positive**	**ALK FISH pattern[§]**	**Abnormal *ALK* copy number/cell[*]**	***ALK* Change**
4	78%	sR	1.2 sR	Positive	90%	sR	1.5 sR	Same
5	37%	split	0.4sR,sG	Positive	51%	split	0.5 sR,sG	Same
6	86%	split	0.8 sR,sG	Positive	70%	split	0.8 sR,sG	Same
7	28%	split	0.3 sR,sG	Positive	82%	split	1.5 sR,sG	**CNG**
8	48%	split	0.5 sR,sG	Positive	66%	split	2.2 sR,sG	**CNG**
9a	80%	sR	1.2sR	**Negative**	2%	NA		**Loss**
9b				Positive	56%	sR	0.9 sR	Same
10[¶]	28%	mix	0.3 sR, 0.2 sG	Positive	30%	mix	0.3 sR, 0.2 sG	Same
11	48%	split	0.5 sR,sG	Positive	56%	split	0.7 sR,sG	Same
12	26%	split	0.3 sR,sG	**Negative**	8%	NA		**Loss**
13[¶]	60%	split	0.6 sR, sG	Positive	48%	split	0.6 sR, sG	Same
14	68%	sR	1.2sR	Positive	92%	sR	1.5 sR	Same

[¶]Patients with intrinsic resistance

[§]sR = single red, sG = single green, split = split red/green, mix = split red/green and single red

[*]Rounded to the nearest tenth decimal

Table 2

Molecular analysis of re-biopsy samples of evaluable patients

	Mechanisms of Resistance				
Patient #	ALK FISH	ALK FISH CNG	ALK kinase domain sequencea	EGFR	KRAS
4	Positive	Negative	**L1196M**	WT[a]	WT[a]
5	Positive	Negative	**L1196M**	WT[a]	WT[a]
6	Positive	Negative	**G1269A**[*]	WT[a,b]	WT[a,b]
7	Positive	**Positive**	**G1269A**	WT[b]	WT[b]
8	Positive	**Positive**	WT	WT[a]	WT[a]
9a	**Negative**	Negative	NA	**L858R**[a,b]	WT[a,b]
9b	Positive	Negative	WT[*]	WT[b]	WT[b]
10[¶]	Positive/Negative[§]	Negative	WT	ND	**G12C**[§b]
11	Positive	Negative	WT	WT[a]	**G12V**[a]
12	**Negative**	Negative	WT	WT[b]	WT[b]
13[¶]	Positive	Negative	WT[*]	ND	ND
14	Positive	Negative	WT	WT[a]	WT[a]

[¶]Patients with intrinsic resistance

[a]Direct sequencing

[b]SNaPshot® includes *APC, AKT1, BRAF, CTNNB1, EGFR, FLT3, JAK2, KIT, KRAS, MAP2K1 (MEK1), NOTCH1, NRAS, PIK3CA, PTEN, TP53*

[*]Heterozygous for rs3795850 *ALK* SNP

[§]From patient-derived cell line with loss of *ALK* gene rearrangement by FISH or RT-PCR .The FFPE tissue was *ALK* FISH +.