Acquired secondary mutations in the anaplastic lymphoma kinase (ALK) gene have been identified in ALK-rearranged (ALK+) non–small-cell lung cancer (NSCLC) patients who developed disease progression while on crizotinib treatment. Here, we identified a novel secondary acquired NSCLC ALK F1174V mutation by comprehensive next-generation sequencing in one ALK+ NSCLC patient who progressed on crizotinib after a prolonged partial response to crizotinib. In a second case, we identified a secondary acquired ALK G1202R, which also confers resistance to alectinib (CH5424802/RO5424802), a second-generation ALK inhibitor that can inhibit ALK gatekeeper L1196M mutation in vitro. ALK G1202R is located at the solvent front of the ALK kinase domain and exhibits a high level of resistance to all other ALK inhibitors currently in clinical development in vitro. Comprehensive genomic profiling of resistant tumor is increasingly important in tailoring treatment decisions after disease progression on crizotinib in ALK+ NSCLC given the promise of second-generation ALK inhibitors and other therapeutic strategies.

Key Words: ALK F1174V, ALK G1202R, Crizotinib resistance, Alectinib, ALK-rearranged NSCLC, Next-generation sequencing.

Acquired secondary resistance mutations to crizotinib in anaplastic lymphoma kinase (ALK)-rearranged (ALK+) non–small-cell lung cancer (NSCLC) has been described.\(^1\)\(^4\) In this brief report, we identified a novel acquired secondary ALK mutation (ALK F1174V) in an ALK+ NSCLC patient who progressed on crizotinib and demonstrated that a previously described secondary mutation, ALK G1202R, confers high level of intrinsic resistance to alectinib, a more potent ALK inhibitor in clinical development, validating in vitro data in a patient for the first time.\(^2\)

Case 1 is a 61-year-old Asian man never-smoker who was diagnosed with stage IV NSCLC in October 2010. Patient was initially treated empirically with erlotinib but had disease progression and received six cycles of carboplatin/pemetrexed/bevacizumab followed by pemetrexed maintenance. Fluorescence in situ hybridization assayed performed was part of screening for a clinical trial of crizotinib, which was positive for the presence of ALK rearrangement but negative for ROS1 rearrangement or MET amplification. Patient was enrolled onto a single-arm phase 2 crizotinib trial (NCT00932451) in May 2011. Repeat magnetic resonance imaging of the brain revealed no brain metastasis at the time of enrollment. He achieved a confirmed partial response after 3 months on crizotinib that lasted 21 months (Fig. 1A). A new 1.8 cm right upper lobe (RUL) pleural nodule that was fluoroodeoxyglucose (FDG)-avid was detected in February 2013 (Fig. 1B). The patient continued on crizotinib for ongoing clinical benefit as he remained asymptomatic. Over the next 6 months, the RUL lobe lesion continued to grow with appearance of a new FDG-avid right lower lobe nodule (Fig. 1C). Computed tomography–guided biopsy of the dominant RUL nodule revealed adenosquamous histology (Fig. 2). Formalin-fixed, paraffin-embedded tissue was submitted to a Clinical Laboratory Improvement Amendments–certified, College of American Pathologist–accredited laboratory, where hybridization capture of 3769 exons of 236 cancer-related genes and 47 introns of 19 genes frequently rearranged in cancer

\[\text{ALK F1174V Mutation and Confirms ALK G1202R Mutation Confers High-Level Resistance to Alectinib (CH5424802/RO5424802) in ALK-Rearranged NSCLC Patients Who Progressed on Crizotinib}\]

*Sai-Hong Ignatius Ou, MD, PhD,*† Michele Azada, MPH,*† David J. Hsiang, MD,*† June M. Herman, MD,§ Tatiana S. Kain, MD,§ Christina Siwak-Tapp, PhD,*† Cameron Casey, BA,*† Jie He, PhD,‖ Siraj M. Ali, MD, PhD,‖ Samuel J. Klempner, MD,*† and Vincent A. Miller, MD‖

*Chao Family Comprehensive Cancer Center, University of California Irvine Medical Center, Orange, California; †Division of Hematology Oncology, Department of Medicine, University of California Irvine School of Medicine, Orange, California; ‡Division of Surgical Oncology, Department of Surgery, University of California Irvine School of Medicine, Orange, California; §Division of Nuclear Medicine, University of California Irvine School of Medicine, Orange, California; and Foundation Medicine Inc, Cambridge, Massachusetts.

Disclosure: Dr. He, Dr. Ali, and Dr. Miller are employees of and have equity in Foundation Medicine, Inc. All other authors declare no conflict of interest.

Address for correspondence: Sai-Hong Ignatius Ou, MD, PhD, Chao Family Comprehensive Cancer Center, University of California Irvine Medical Center, 101 City Drive, Bldg 56, RT51, Rm 241, Orange, CA 92868–3298. E-mail: Ignatius.ou@uci.edu

Copyright © 2014 by the International Association for the Study of Lung Cancer

ISSN: 1556-0864/14/0904-0549
was applied to 200 ng of DNA extracted from this sample and sequenced to high, uniform coverage of 939x (Foundation One, Foundation Medicine Inc., Cambridge, MA). This assay confirmed the presence of EML4-ALK variant 3 (E6; A20) and identified the presence of a novel ALK F1174V mutation (Fig. 3A) and quantitatively estimated the ALK F1174V allele frequency at 23% in a background of 40% tumor purity, consistent with a clonal heterozygous substitution; a S45 deletion in CTNNB1 was also detected. The patient is now enrolled in a phase 2 trial of alectinib (RO5424802/CH5424802) for crizotinib-resistant ALK+ NSCLC patients (NCT01588028) at 600 mg orally twice a day (the recommended phase 2 dose).

Case 2 is a 60-year-old Asian woman never-smoker who was diagnosed with stage IV adenocarcinoma of the lung NSCLC in June 2010. She received four cycles of carboplatin/albunin bound-paclitaxel/bevacizumab chemotherapy followed by pemetrexed/bevacizumab maintenance chemotherapy from July 2010 to December 2010 but had disease progression in January 2011. Fluorescence in situ hybridization assay on her tumor was positive for ALK rearrangement and negative for ROS1 rearrangement or MET amplification. She was enrolled on a single-arm phase 2 crizotinib trial (NCT00585195) in February 2011 and achieved a confirmed partial response after 3 months of crizotinib treatment (RECIST 1.0)5 (Fig. 2A and B). Twenty months into crizotinib treatment, she developed RECIST-defined disease progression of 20% increase in aggregate tumor measurement from the lowest achieved baseline achieved due to an enlarging right hilar lymph node. Simultaneously, a right axillary lymph node demonstrated increasing FDG avidity with a standardized uptake value (SUV) of 2.0 (Figs. 1C and 2A) but was still normal in size (<1 cm). She was continued on crizotinib post progression due to ongoing clinical benefit as she was asymptomatic and her aggregate tumor measurement was still at 63% reduction from the precrizotinib treatment level. The right axillary lymph node (LN) continued to grow in size to 1.1 cm and the SUV increased to 7.8 (Fig. 2D). Crizotinib was discontinued and patient was

![FIGURE 1. A, Partial response to crizotinib 12 months after crizotinib treatment. B, Appearance of a new right upper lobe pleural-based nodule after 26 months of crizotinib treatment. C, Continual growth of the right upper lobe pleural-based nodule and a new right lower lobe nodule after 32 months of crizotinib treatment.](image-url)
enrolled onto the same alectinib trial as patient 1. Pre-enrollment the right axillary LN measured 1.5 cm with a SUV of 8.9 (Fig. 2E). Restaging workup did not reveal any brain metastasis. She started alectinib 600 mg orally twice a day on May 2013. Nevertheless, disease progression continued after 5 weeks on alectinib, as evidence the right axillary LN had increased in size to 2.0 cm with a SUV of 11.9 (Fig. 2F). RECIST progression was confirmed 9 weeks (Fig. 2G) after starting alectinib due to continued growth of the right axillary LN to 2.2 cm with a SUV of 11.0. Patient also had pain around the right axillary lymph node, which was completely excised for symptoms relief. Fifteen 4-μm-thick unstained slides from the formalin-fixed paraffin-embedded lymph node were run on the Foundation One test. The assay confirmed the presence of an EML4-ALK rearrangement (E6; A20) (variant 3) and an ALK G1202R mutation with an estimated allele frequency of 23% in a background of 60% tumor purity, consistent with a near clonal heterozygous substitution (Fig. 3B). In addition, loss of CDKN2A/B and ARFRP1 amplification (9X) were identified. No EGFR or KRAS alterations or ALK amplification was identified. The patient’s original tumor tissue was exhausted during the Lung Cancer Mutation Consortium profiling effort. Patient’s right chest pain was resolved post axillary lymph node excision and continued on alectinib post progression as allowed by protocol but had continued disease progression (Fig. 2H). Of note, both patients’ tumor sample was profiled using the SNaPSHOT analysis of 15 genes (APC, AKT1, BRAF, CTNNB1, EGFR, FLT3, JAK2, KRAS, MEK1, NOTCH1, NRAS, PIK3CA, PTEN, TP53) and was negative for base substitutions at mutational “hotspots”; notably, ALK sequencing is not a component of this analysis.

**DISCUSSION**

To date, the major mechanisms of crizotinib resistance in ALK+ NSCLC patients include acquired secondary resistance mutations in the kinase domain, amplification of the ALK fusion gene, and bypass track activation. Using a targeted comprehensive next-generation sequencing approach, we have identified a novel ALK F1174V mutation in an ALK+ NSCLC patient resistant to crizotinib. Previously, an ALK F1174L mutation has been identified as an acquired secondary resistance mechanism to crizotinib in an inflammatory myofibroblastic tumor patient harboring RANBP2-ALK rearrangement who progressed on crizotinib. ALK F1174L is also a common activating mutation in sporadic neuroblastoma, but rare base substitutions in the other two codons encoding
phenylalanine at residue 1174 (F1174V, F1174C) have been also been reported in neuroblastoma.\textsuperscript{9} In vitro chemical mutagenesis experiments in the setting of increasing crizotinib dose have identified phenylalanine at amino acid residue 1174 as a common site of secondary resistance to crizotinib and involving all three positions of the codon encoding phenylalanine (F1174L/C/V/I).\textsuperscript{10} Earlier to this report, there are no reports identifying base substitutions within the codon of phenylalanine at amino acid residue 1174 as a secondary acquired crizotinib resistance mechanism in NSCLC. The RANBP2-ALK F1174L mutation lies near the C-terminus of the αC helix, and its side chain is involved in the hydrophobic core adjacent to the activation loop of the ALK protein. The leucine (L) for phenylalanine (F) alteration is thought to reduce the side chain size which weakens the normal autoinhibitory interactions thereby allowing ALK F1174L to favor the active conformation\textsuperscript{11} thereby increasing the ATP-binding affinity of ALK and basal activation of ALK \textsuperscript{8,11,12} without increasing its affinity for crizotinib, with a net effect of reducing crizotinib sensitivity. As both valine (V) and leucine (L) are branched chain hydrophobic amino acids, we hypothesize that the resistance mechanism of ALK F1174V is similar to ALK F1174L. Indeed, neuroblastoma cell lines harboring the ALK F1174V mutation (CHLA90, CLB-GE) ALK kinase activity are increased.\textsuperscript{13,14} In vitro cell-based experiments have shown that ALK inhibitors with higher affinity for ALK kinase domain (alectinib, LDK378, AP26113) can overcome the ATP-binding kinetics of ALK F1174L.\textsuperscript{15} In contrast,
ALK G1202R, which has been previously described in a patient who progressed on crizotinib, is highly resistant to all ALK inhibitors in clinical development (LDK378, alectinib, ASP3026, AP26113) although the PK level achieved at the recommended dose of AP26113 (180 mg qD) is above the concentration needed to inhibit 50% of (IC$_{50}$) ALK G1202R. ALK G1202R is postulated to be on the solvent-exposed region of the ALK kinase domain abutting the crizotinib-binding site and likely diminishes the binding affinity of crizotinib and other ALK inhibitors to the mutant ALK from steric hindrance due to the presence of a large basic residue. We are not certain whether the G1202R mutation arose following crizotinib resistance or alectinib therapy, although the timing of disease progression on alectinib suggests that it was present at the time of crizotinib resistance.

It is unknown the significance of role of S45 deletion in the CTNNB1 in the background of ALK F1174V or loss of CDKN2A/B and ARFRP1 amplification in the background of ALK G1202R in conferring resistance to crizotinib. Nevertheless, targeted next-generation sequencing offers comprehensive genomic profiling allowing the identification of population of resistant tumor clones and other underlying genetic alterations beyond secondary acquired mutations thus offering increased granularity in one diagnostic setting allowing even the small amount of tumor obtained from rebiopsy to yield significant useful clinical information.

ACKNOWLEDGMENT

We thank Phil Stephens, PhD, at Foundation Medicine for reviewing the manuscript.

REFERENCES