



## ORIGINAL ARTICLE

# Heterogeneous responses and resistant mechanisms to crizotinib in *ALK*-positive advanced non-small cell lung cancer

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## Keywords

*ALK*; DNA mismatch repair; next-generation sequencing; NSCLC; resistance.

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Received: 26 April 2018;

Accepted: 24 May 2018.

doi: 10.1111/1759-7714.12791

Thoracic Cancer 9 (2018) 1093–1103

## Abstract

**Background:** *ALK*-tyrosine kinase inhibitors (TKIs) have been proven effective for treating *ALK*-positive non-small cell lung cancer (NSCLC), although patients present with variable responses and disease progression courses. The detailed underlying molecular mechanisms require further investigation to yield a better prognosis.

**Methods:** Targeted next-generation sequencing (NGS) mutation profiling was performed on samples from 42 NSCLC patients confirmed positive for *ALK* rearrangements by fluorescence in situ hybridization or immunohistochemistry who experienced disease progression after crizotinib treatment.

**Results:** *ALK* rearrangements were not confirmed in six patients (14%) with other potential oncogenic drivers identified by NGS, who therefore did not respond to crizotinib and had significantly shorter overall survival (OS) compared to NGS *ALK* -positive patients. Fifteen *ALK* activating mutations were detected in 8 out of 26 post-treatment samples (31%), among which *ALK* L1196M and G1269A were the most common acquired mutations detected in half of the patients with *ALK* activating mutations. Dynamic monitoring of the genetic evolution in one patient revealed both spatial and temporal heterogeneity of resistant mechanisms during different *ALK*-TKI treatment courses. Activation of *ALK* downstream or bypass pathways was detected in patients without *ALK* activating mutations, such as genetic alterations in *PIK3CA*, *MET*, and *KRAS*. Interestingly, we identified two patients with acquired mutations in the DNA mismatch repair gene *POLE*, which resulted in a dramatically increased tumor mutation burden, and might contribute to the poor response to crizotinib.

**Conclusions:** Heterogeneous resistant mechanisms have been identified and correlate to diverse responses to crizotinib. Comprehensive and dynamic mutation profiling is required to better predict clinical outcomes.

## Introduction

ALK rearrangements occur in approximately 3–7% of non-small cell lung cancers (NSCLCs).<sup>1</sup> The most common fusion partner of ALK is *EML4*, in which several variants with different break points in *EML4* have been discovered.<sup>2</sup> In two pivotal and randomized phase III trials, crizotinib showed superiority to standard cytotoxic chemotherapy in NSCLC, which established crizotinib as first-line therapy for ALK-rearranged patients.<sup>3,4</sup> Crizotinib significantly improves objective response rates, progression-free survival (PFS), quality of life, and particularly overall survival (OS). However, although the efficacy of crizotinib is remarkable, patients with an initial response inevitably develop resistance to crizotinib within one to two years.<sup>5</sup> Mechanisms of resistance include acquired mutations in ALK, such as ALK L1196M and G1269A point mutations, which hinder the binding of crizotinib; activation mutations in ALK downstream signaling pathways, such as the activation of *KRAS* and *PIK3CA*; overexpression of ALK-fusion proteins; and activation of ALK bypass signaling pathways, such as *EGFR*, *MET*, and *HGF*. In the remaining patients, no known mechanisms of secondary resistance have been elucidated.<sup>6,7</sup> To overcome the resistance of ALK activating mutations to crizotinib, second and third-generation ALK-TKIs, including ceritinib, alectinib, brigatinib, and lorlatinib, have recently been developed for the subsequent treatment of patients carrying secondary ALK mutations and have demonstrated more potent and significant antitumor activity.

In order to explore the mechanisms of resistance to crizotinib, we conducted a retrospective study including 42 patients after an initial screening of 270 ALK break apart fluorescent in situ hybridization (FISH)-positive NSCLC patients. Next-generation sequencing (NGS) was performed and mutation profiles of pre-treatment and post-treatment samples were analyzed to provide possible guidance for therapeutic strategies after ALK-TKI treatment.

## Methods

### Patients and samples

From October 2010 to December 2017, 270 patients from Guangdong General Hospital were diagnosed with ALK-positive NSCLC. All patients were diagnosed with locally advanced or metastatic NSCLC according to the 2009 International Association for the Study of Lung Cancer staging system (version 7).<sup>8</sup> Pathohistological classification of tumor type was performed with formalin-fixed, paraffin-embedded (FFPE) tumor samples collected upon diagnosis. All patients received crizotinib as first-line ALK-TKI treatment and some received subsequent ALK-TKIs as further

treatment. Clinical responses were evaluated via computed tomography (CT) scans six weeks after the first administration of crizotinib and every six weeks thereafter according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1.<sup>9</sup> PFS was measured from the date of initiation of crizotinib treatment until disease progression or death. Overall survival (OS) was calculated from the date of NSCLC diagnosis to death resulting from any cause or was censored at the last follow-up in December 2017. Repeat biopsies were performed in some cases for pathohistological confirmation of metastatic sites. Blood samples were regularly collected to monitor tumor markers and/or circulating tumor DNA (ctDNA).

The institutional ethic review board of Guangdong General Hospital approved the study. All patients enrolled provided written informed consent for specimen collection, genetic testing, and to use their data for research purposes.

### Detection of ALK rearrangements with ALK break apart fluorescence in situ hybridization and/or immunohistochemistry

Unstained FFPE sections from tumor specimens collected at diagnosis were subjected to FISH with ALK break apart probes (Vysis ALK Break Apart FISH Probe Kit; Abbott Molecular, Abbot Park, IL, USA) and/or immunohistochemistry (IHC) staining with Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions.

### DNA extraction, library preparation, and targeted next-generation sequencing (NGS)

Tumor genomic DNA was extracted from FFPE sections with a tumor content > 50% using a QIAamp DNA FFPE Kit (Qiagen, Hilden, Germany) to detect somatic mutations. Available plasma samples were used to extract ctDNA using a QIAamp Circulating Nucleic Acid Kit (Qiagen) with optimized manufacturer protocols. Each sample type is listed in Table S1. Of the 27 pre-treatment samples, 7 were plasma ctDNA specimens, while 6 and 1 out of the 28 post-treatment samples were ctDNA from plasma and cerebrospinal fluid specimens, respectively. Genomic DNA was extracted from whole blood specimens of each patient using a Gentra Puregene Blood Kit (Qiagen), which was then used to subtract germline mutations in the subsequent NGS result analysis pipeline.

Hybridization capture-based targeted NGS was performed at two genetic testing centers for 382 cancer-

relevant genes and 16 fusion genes frequently rearranged in solid tumors,<sup>10</sup> 287 cancer-related genes and introns from 22 genes frequently rearranged in tumors, or 61 genes and mutation hotspots in lung cancer from 107 cancer-relevant genes on Illumina HiSeq4000 NGS platforms (Illumina Inc., Madison, WI, USA). The panel used for each sample is listed in Table S1.

### NGS data processing and identification of *ALK* rearrangement

After demultiplexing, FASTQ files were processed with Trimmomatic for quality control,<sup>11</sup> and then mapped to human reference genome 19 (hg19) using Burrows-Wheeler Aligner.<sup>12</sup> A Genome Analysis Toolkit was used for local realignment and base quality score recalibration.<sup>13</sup> Single nucleotide variation (SNV) and indel mutation calling was performed using VarScan (<http://dkoboldt.github.io/varscan/>) for minor allele frequency (MAF) < 10%, or Genome Analysis Toolkit for MAF > 10%. Somatic mutations were first filtered for common SNPs with dbSNP and 1000 Genome datasets, followed by further filtration of germline mutations with normal blood controls. Structural variants were detected using FACTERA with default parameters,<sup>14</sup> and ADTEX (<http://adtx.sourceforge.net>) was used to identify copy number variations (CNV) with default parameters.

### Statistical analysis

A Cox proportional hazards model was applied to assess whether there was an association between baseline characteristics and PFS of crizotinib. The Gehan-Breslow-Wilcoxon method was used to assess the significance of PFS and OS curves of different patient groups after crizotinib treatment. A *P* value of < 0.05 was considered statistically significant. Hazard ratios (HR) were calculated using the Mantel-Haenszel method.

## Results

### Patient enrolment

From October 2010 to December 2017, 270 patients were diagnosed with *ALK*-positive NSCLC based on Vysis *ALK* Break Apart FISH or *ALK* IHC results at the Guangdong General Hospital. All 270 patients were orally administered 250 mg crizotinib twice, and 182 experienced disease progression (Fig S1). Among the 182 patients, 42 patients with samples (either FFPE sections or plasma) available for NGS testing were enrolled in the study. In this patient cohort, half of the patients (*n* = 21, 50%) achieved PFS with crizotinib of ≤ 3 months, while 21 patients (50%)

achieved PFS with crizotinib > 3 months. Notably, according to NGS results, *ALK* rearrangement was not detected in six patients (14%) who achieved PFS < 3 months on crizotinib (Fig S1).

The baseline clinicopathologic characteristics of the 42 patients enrolled in this study are listed in Table S2. Crizotinib was administered to 18 patients (43%) as first-line treatment, to 16 (38%) as second-line treatment, and to 8 (19%) as a third-line or further treatment.

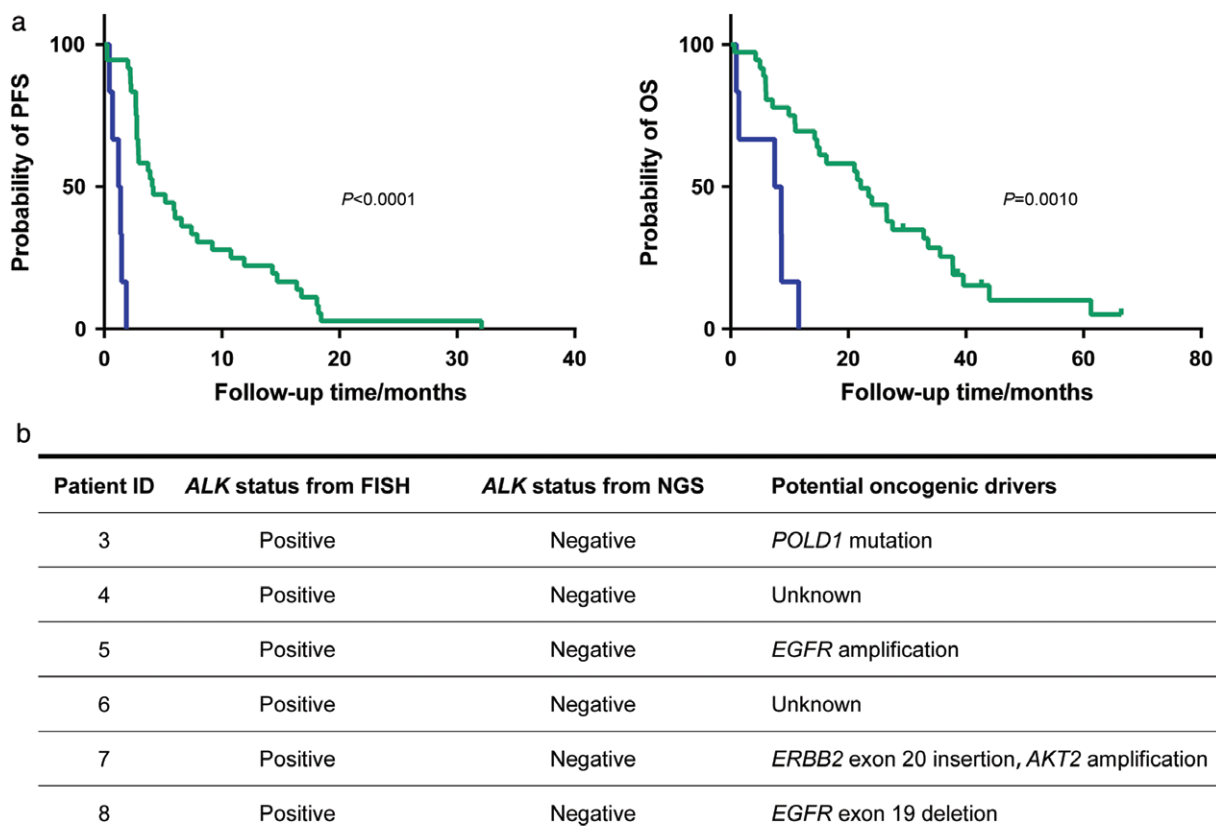
### NGS *ALK*-negative patients had significantly shorter PFS and OS than *ALK*-positive patients

Based on NGS results, *ALK* rearrangement was not detected in six patients (14%), although all showed split signals via *ALK* break apart FISH. These patients had significantly poorer clinical outcomes on crizotinib compared to patients with NGS *ALK*-positive results. As shown in Figure 1a, the median PFS on crizotinib was significantly longer (HR 1217, 95% confidence interval [CI] 122.2–12 123; *P* < 0.0001) in NGS *ALK*-positive patients (median PFS 4.2 months, 95% CI 2.8–7.9) than in NGS *ALK*-negative patients (median PFS 1.3 months, 95% CI 0.4–1.9). The OS of NGS *ALK*-positive patients (median OS 21.8 months, 95% CI 14.27–29.30) was significantly longer than that of NGS *ALK*-negative patients (median OS 8.0 months, 95% CI 1–11.6) (HR 20.42, 95% CI 4.009–104.1; *P* = 0.001). We further analyzed potential oncogenic drivers in NGS *ALK*-negative patients based on NGS results (Fig 1b). Among the six patients, two were *EGFR* positive, including one with an *EGFR* amplification (patient 5) and one with an exon 19 deletion (patient 8). Patient 7 carried an *ERBB2* exon 20 insertion, which is a well known driver in lung adenocarcinoma.<sup>15</sup>

*ALK* rearrangement was detected in the remaining patients according to NGS results. The distribution of *ALK* fusion partners is listed in Table S3. Seven (17%) patients carried non-*EML4-ALK* fusion partners and 29 (69%) *EML4-ALK*, including 7 v1 (17%), 15 v3 (36%), and 7 other variants (17%).

### Genetic profiles of baseline samples

The genetic alterations detected via NGS are listed in Table S4. Figure 2 shows the mutation profiles of the 27 pre-treatment samples. According to the genetic profiles of *ALK*-positive patients, *TP53* was the most frequently mutated gene, followed by *ALK* rearrangement. Other mutated genes with recurrence ≥ 2 in the *ALK*-rearranged patients included *SETD2*, *RBI*, *CREBBP*, and *ATM*. No difference was observed in the mutation frequency of these



**Figure 1** Differential clinical outcomes to crizotinib according to ALK fusion status confirmed by next-generation sequencing (NGS). (a). Kaplan-Meier curves of progression-free survival (PFS) after crizotinib and overall survival (OS) of ALK fusion positive or negative patients determined by NGS. (—) NGS ALK-negative ( $n = 6$ ) and (—) NGS ALK-positive ( $n = 36$ ). (b). Potential driver genetic alterations in NGS ALK-negative patients. The  $P$  value between PFS and OS curves was calculated according to the Gehan-Breslow-Wilcoxon test. FISH, fluorescence in situ hybridization.

genes between patients with PFS  $\leq 3$  months and  $> 3$  months according to Fisher’s exact test.

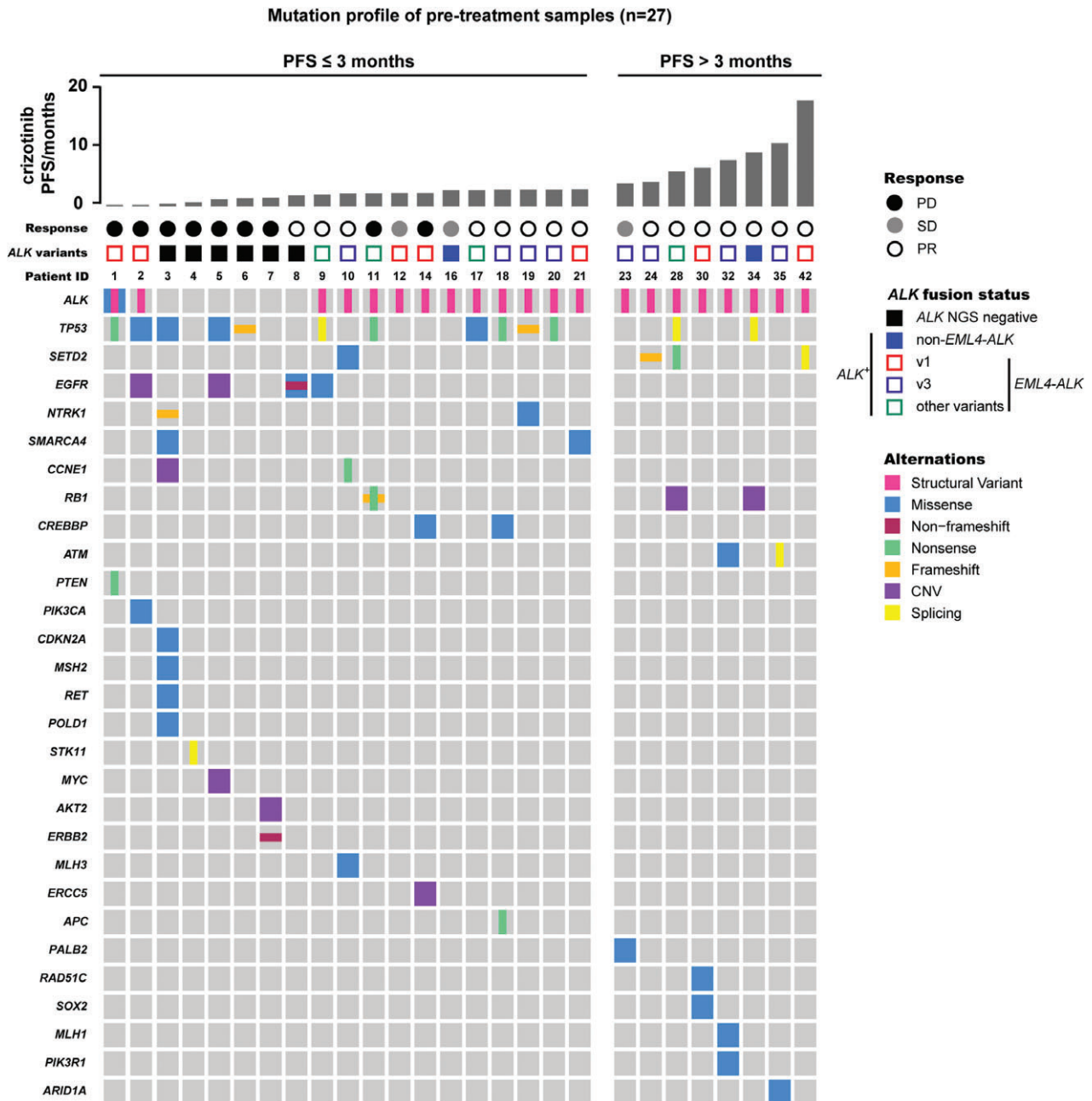
Notably, a pre-existing ALK E1303K mutation was detected in patient 1 (Fig 1, Table S4), who experienced disease progression after crizotinib treatment and PFS of seven days. To investigate if this mutation was associated with the poor clinical outcome, we reconstructed the 3D structure of the ALK E1303K mutation based on the crystal structure of the kinase domain of ALK using PyMOL.<sup>16</sup> As shown in Figure S2, the point mutation is located from a distance of the crizotinib binding pocket with an unknown effect on protein structure; thus, the effect of E1303K on response to crizotinib and other ALK-TKIs should be further validated.

### ALK activating mutations detected in post-treatment samples

Post-treatment samples from 26 patients in our cohort were available for NGS (Fig 3). ALK activating mutations were detected in eight patients (19%), five of which were acquired during the treatment process by comparing paired

samples taken pre-treatment and post-treatment (Fig 4). Figure 5a shows the details of ALK activating mutations in this patient cohort. ALK L1196M and G1269A were the most frequent activating mutations, which were detected in four patients, respectively. Notably, the four patients harboring L1196M had PFS on crizotinib  $< 6$  months.

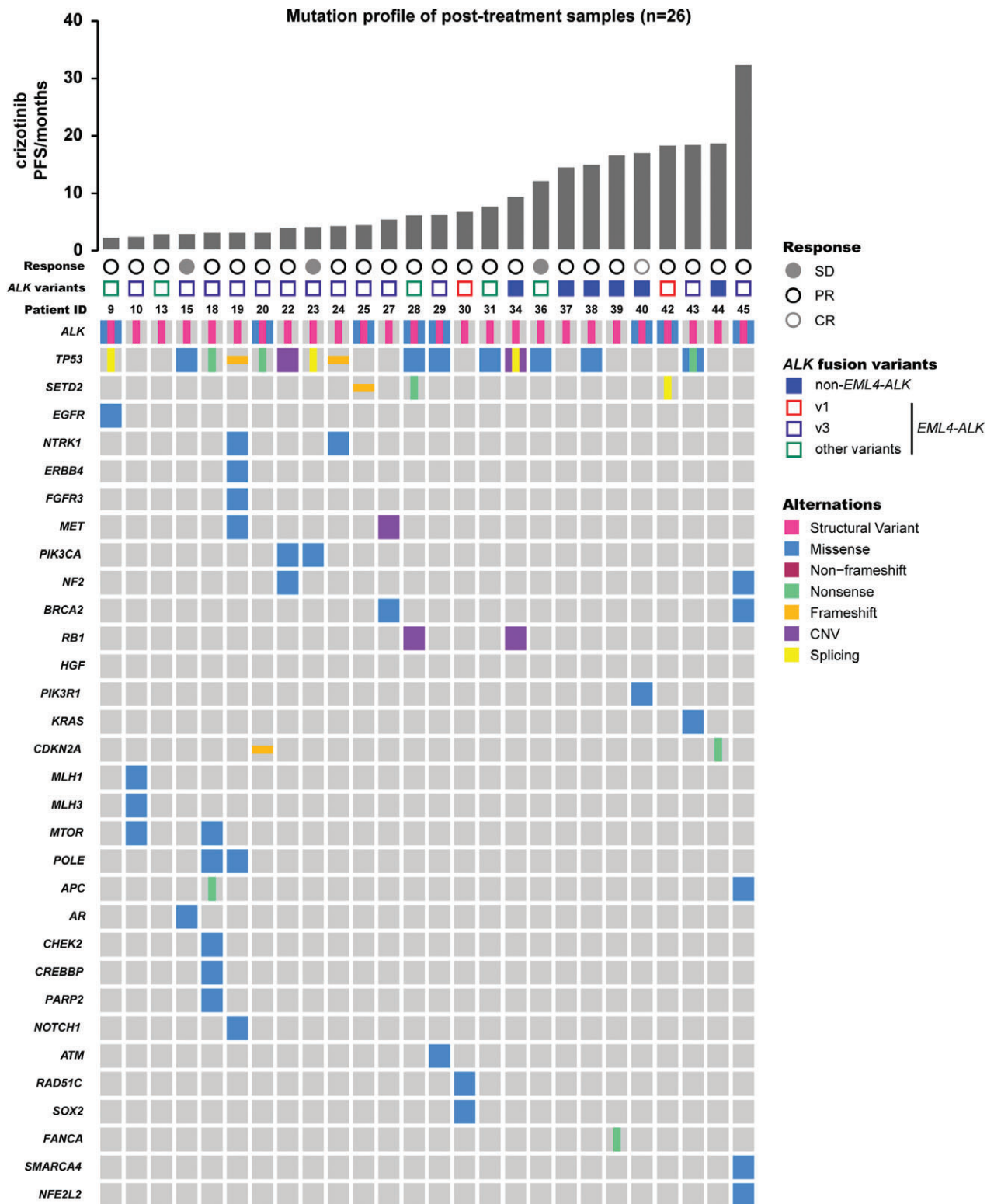
The co-existence of multiple ALK activating mutations was detected in four samples from three patients. As shown in Figure 5b, dynamic samples from patient 45 were subjected to NGS analysis, including a sample from a brain metastasis nodule (45a) taken after the administration of alectinib treatment for three months and plasma (45b) and cerebrospinal fluid (CSF) samples (45c) taken at the end of alectinib treatment. Four ALK activating mutations were detected in 45a, which was harboring the most ALK activating mutations in this patient cohort, including G1269A, F1174L, E1154Q, and E1210K. Thus, we further performed allelic discrimination analysis of samples containing multiple ALK activating mutations. In 45a, no reads encompassing F1174L and E1210K mutations were found in the NGS results, suggesting that these two mutations are not located on the same allele.



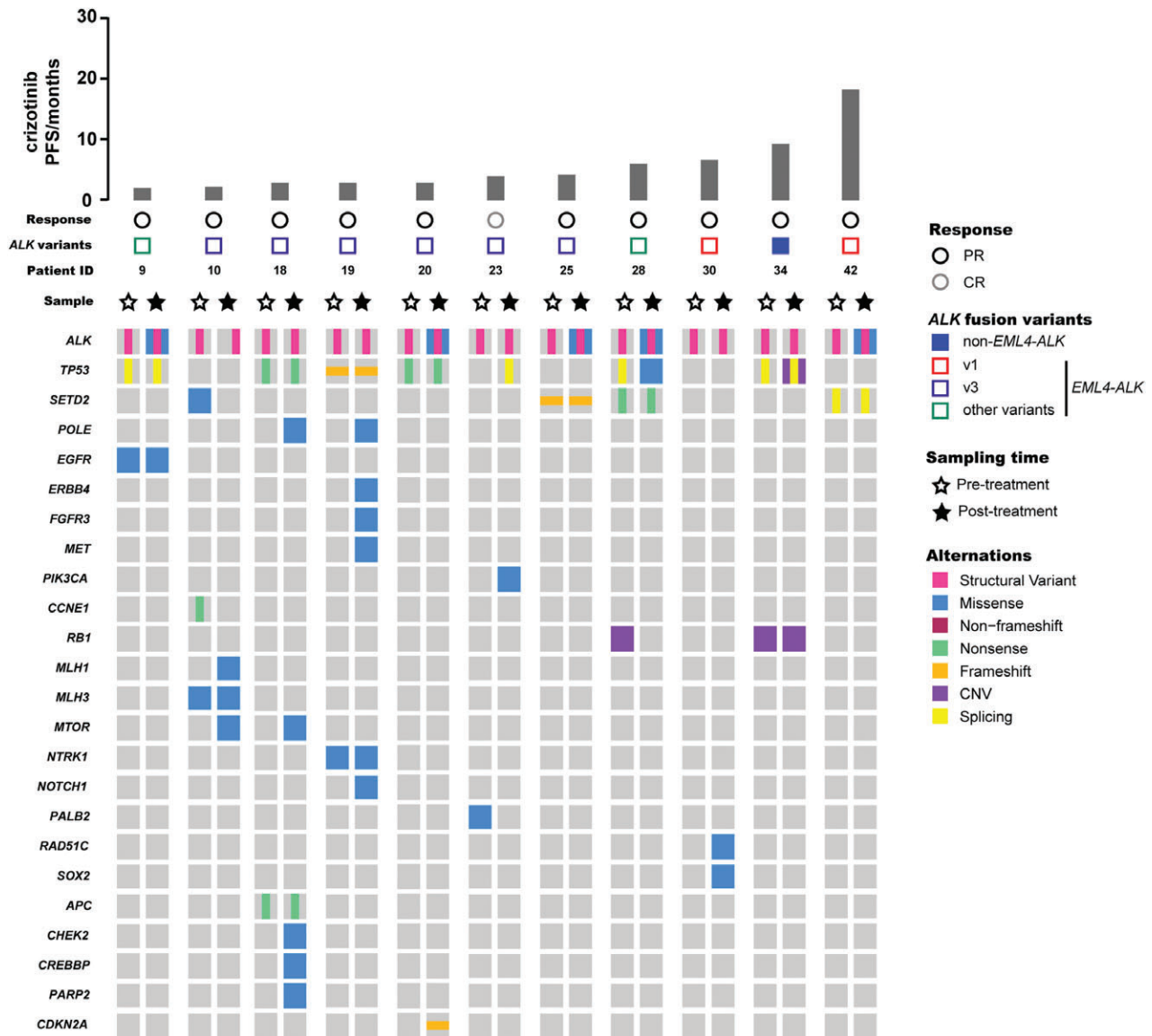
**Figure 2** Genetic profiles of pre-treatment samples of non-small cell lung cancer patients positive for ALK via break apart fluorescence in situ hybridization or immunohistochemistry test with variable responses to crizotinib. Patients were stratified into two groups based on their progression-free survival (PFS) after crizotinib treatment, with PFS for each individual shown as a bar graph (top). Mutated genes identified in at least two patients are shown and ranked by their recurrence frequency. Patients' response to crizotinib, ALK fusion status, and types of genetic alterations in each gene are indicated accordingly. CNV, copy number variation; PD, progressive disease; PR, partial response; SD, stable disease.

Although we could not confirm the allelic discrimination of other co-existing mutations from the NGS data because of the separation of introns between mutation sites, based on the different allele frequency of co-existing mutations

detected in each sample, it is likely that these co-existing mutations are located on different alleles in samples 9, 29, and 45b. These data suggest both spatial and temporal tumor heterogeneity obtained during the development of



**Figure 3** Genetic profiles of post-treatment samples from patients who experienced disease progression on crizotinib. Patients were ranked according to their progression-free survival (PFS) shown as a bar graph (top). Mutated genes identified in at least two patients are shown and ranked by their recurrence frequency. Patients' response to crizotinib, ALK fusion variants, and types of genetic alterations in each gene are indicated accordingly. CNV, copy number variation; PD, progressive disease; PR, partial response; SD, stable disease.



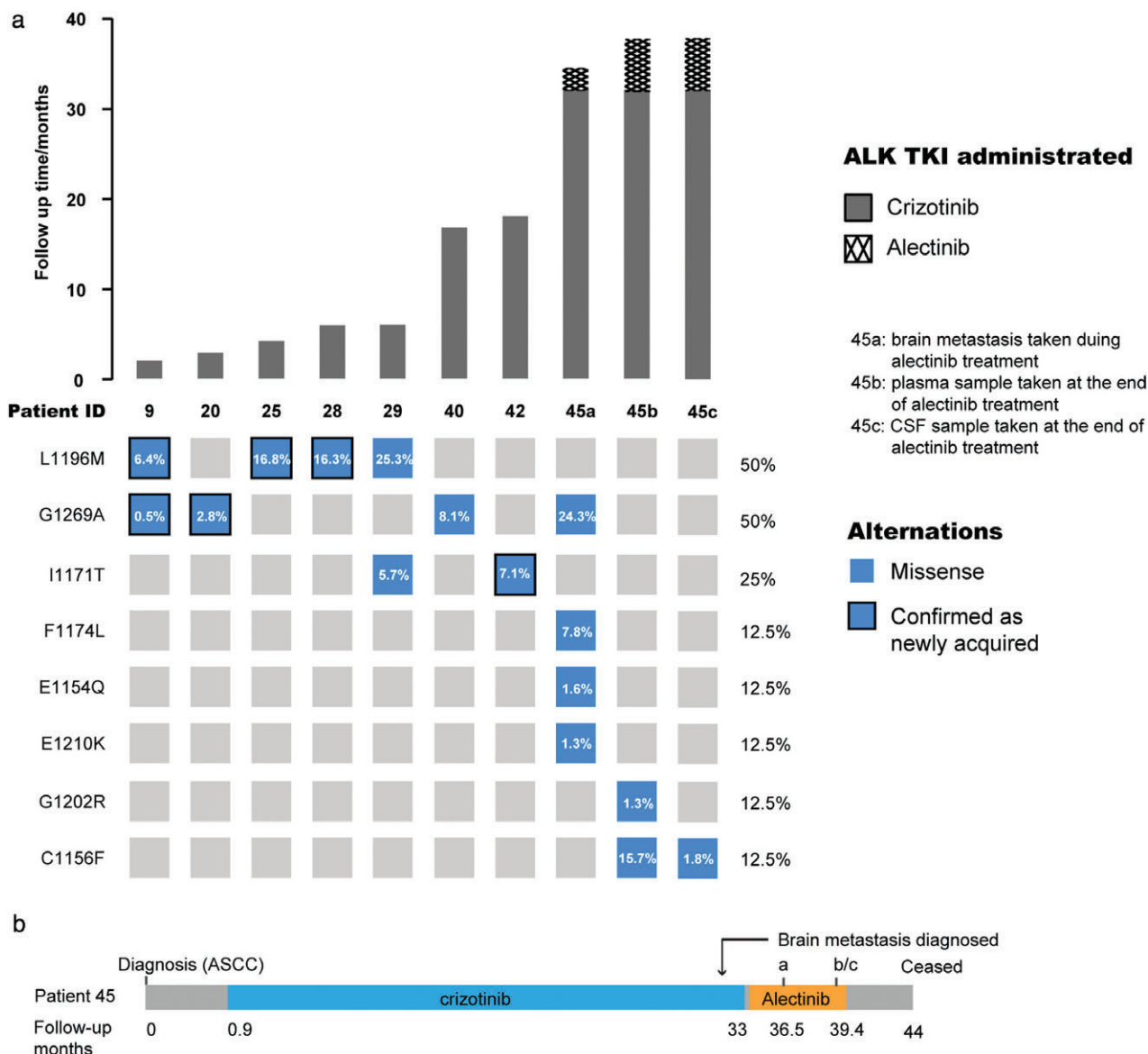
**Figure 4** Matched pre-crizotinib and post-crizotinib treatment samples reveal newly acquired genetic alterations during disease progression. Patients were ranked according to their progression-free survival (PFS), shown as a bar graph (top). Patients' response to crizotinib, ALK fusion variants, and types of genetic alterations in each gene are indicated accordingly. CNV, copy number variation; PD, progressive disease; PR, partial response.

resistance to ALK-TKIs. The only exception is that it is difficult to conclude the *in trans* configuration of E1154Q and E1210K in patient 45 because of the similar allele frequency.

In patients 9 and 20, L1196M/G1269A and G1269A were only present in the post-treatment samples, respectively (Figs 4–5). As no such mutations were detected in their baseline samples, it is likely that these mutations were rapidly acquired within the three months during crizotinib treatment, although we cannot discount the possibility that these activating mutations were not detected in the baseline samples because of low MAF or different sampling sites.

### Activation of ALK downstream and bypass pathways in post-treatment samples

Activation of ALK downstream pathways were detected in three patients, including *PIK3CA* R108H in patient 22, *PIK3CA* E542K in patient 23, and *KRAS* Q61K in patient 45 (Fig 3, Table S4). *PIK3CA* E542K was confirmed as newly acquired after crizotinib treatment (Fig 4). Furthermore, activation of ALK bypass pathways was also detected in this patient cohort, including copy number gains of *EGFR* and *HGF* (patient 33) and *MET* (patient 27). Of note, an *EGFR* P596L mutation, which has been reported in glioblastoma,<sup>17</sup> was present in both



**Figure 5** Frequency of *ALK* activating mutations detected in post-treatment samples. **(a)** *ALK* activating mutations identified in eight patients after *ALK*-tyrosine kinase inhibitor (TKI) treatment. The mutant allele frequency (MAF) of each mutation is shown accordingly in the co-mutation plot. Three samples taken at two time points from patient 45 were subjected to next-generation sequencing (NGS), as indicated. **(b)** Treatment timeline of patient 45 showing the sampling time for NGS. ASCC, adenocarcinoma; CSF, cerebrospinal fluid.

pre-treatment and post-treatment samples in patient 9. Thus, it is of interest to further explore the co-existence of *ALK* rearrangements and *EGFR* P596L mutations, along with the acquisition of *ALK* activating mutations (L1196M/G1269A) after crizotinib treatment, as well as validating the oncogenicity of *EGFR* P596L mutations.

In the paired samples, mutations in *POLE*, encoding  $\epsilon$  catalytic subunit in DNA polymerase were observed in patients 18 and 19, which are associated with a dramatic increase in the total number of somatic mutations, and

were acquired after crizotinib treatment (Fig 6a). Particularly in patient 19, in addition to *POLE*, a series of missense mutations were detected in *ERBB4* A648T, *FGFR3* A106V, and *MET* A1004V (Table S4), none of which were reported in COSMIC, suggesting that these mutations are likely passenger mutations resulting from DNA mismatch repair (MMR) deficiency in this patient. These two patients had mixed clinical outcomes to crizotinib treatment. As shown in Figure 6b, multiple metastases were detected at diagnosis in patient 18 at the paracaval lymph nodes and on the right adrenal gland (Fig 6b), and



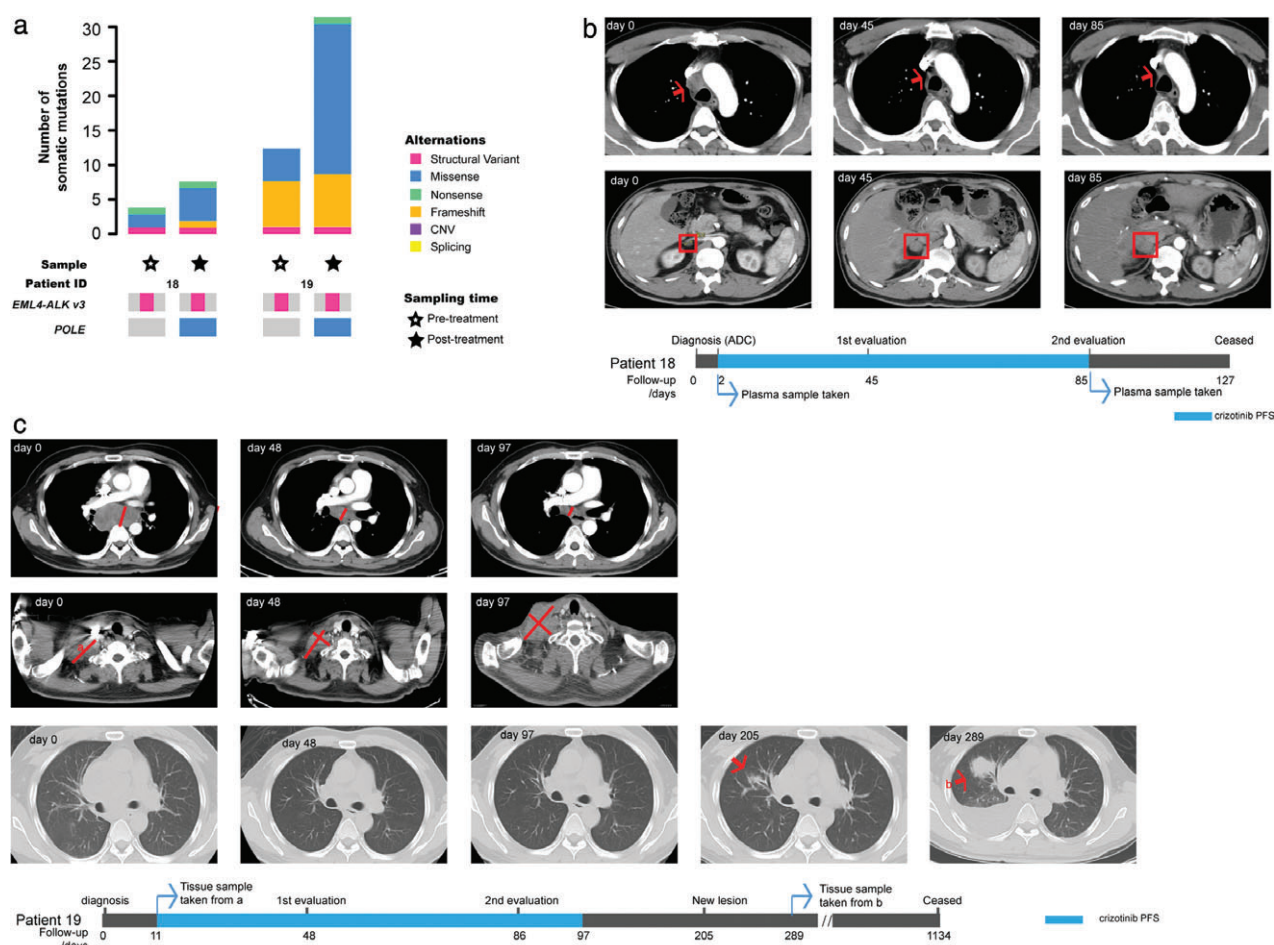
crizotinib was administered immediately after FISH *ALK*-positive results were obtained. Although a partial response was achieved in the paracaval lymph nodes, disease progression was observed in the right adrenal gland at the first and second response evaluation. In patient 19, multiple metastatic sites were detected at diagnosis and mixed effects were reached after crizotinib treatment, that is, a sustained increase of the supraclavicular lymph node metastasis (where the pre-treatment sample was taken) versus a reduction of the inferior tracheobronchial lymph node metastasis (Figure 6c). After the cessation of crizotinib treatment, a new lesion was detected in the right lung on day 205 and showed continuous enlargement on day 289 when a post-treatment sample was taken. These results suggest that rapidly acquired *POLE* mutations may be a

resistance mechanism to crizotinib treatment and might indicate poor clinical outcomes.

### Discussion

We retrospectively analyzed mutational profiles in both baseline and post-treatment samples from NGS results in *ALK*-positive NSCLC patients and revealed both spatial and temporal heterogeneity of resistant mechanisms during the treatment courses with different *ALK*-TKIs, which may provide guidance for better clinical care and improved outcomes.

In this patient cohort, *EML4-ALK* v3 was the most common *ALK* fusion variant, detected in 15 patients (36%). Although it has been reported that different *EML4-ALK*



**Figure 6** Association of DNA mismatch repair (DMMR) deficiency and poor clinical outcomes in patients with acquired *POLE* mutations. (a) *POLE* mutations were detected in the post-treatment samples of patients 18 and 19, which are associated with dramatic gain of missense mutations. (b) Disease course and computed tomography (CT) images of patient 18 show shrinkage of the paracaval lymph nodes (top panel, red arrow) and gradual enlargement of a metastatic nodule on the right adrenal gland (bottom panel, red box). (c) Disease course and CT images of patient 19 showing mixed effects. Metastasis of the inferior tracheobronchial lymph node was evaluated as a partial response (top panel, red line for measurement). Disease progression was observed at the supraclavicular lymph node (middle panel, red cross for measurement). A new lesion was detected in the right lung six months after cessation of crizotinib (bottom panel). ADC, adenocarcinoma.

variants show different clinical outcomes to ALK-TKI treatment,<sup>18,19</sup> we did not observe any differences among different *EML4-ALK* variants in this patient cohort (data not shown).

Acquired *POLE* mutations were detected in post-treatment samples, along with high mutation number gains within three months when pre-treatment samples (patients 18 and 19) were compared during crizotinib treatment (Fig 6). *POLE*, encoding  $\epsilon$  catalytic subunit in DNA polymerase, is involved in DMMR.<sup>20</sup> Both patients 18 and 19 had PFS < 3 months after crizotinib treatment, suggesting that *POLE* mutations could be rapidly acquired. Once mutations in DNA polymerases occurred, passenger mutations resulting from DMMR deficiency accumulated and may cause resistance to crizotinib.

In addition to investigating the molecular mechanism of resistance to crizotinib in ALK-positive NSCLC patients, this study also investigated the therapeutic strategies according to these findings for best patient benefit. In this patient cohort, discrepancies were found in six patients with positive results in ALK break apart FISH but negative results in NGS, including two *EGFR*-positive and one *ERBB2*-positive from the pre-treatment samples. With a poor response and short PFS, these patients did not benefit from crizotinib treatment. Instead, the three *EGFR/ERBB2* positive patients may gain benefits from corresponding EGFR-TKIs or anti-ERBB2 therapy if NGS testing were performed at diagnosis.

After the development of resistance to crizotinib, NGS results of repeat biopsy samples or ctDNA from liquid biopsy for dynamic monitoring could provide further guidance for subsequent therapeutic strategies. Synergistic combined treatments, such as ALK-TKIs combined with *PIK3CA/KIT/MTOR/MYC/EGFR* inhibitors, are a promising therapeutic strategy in clinical practice for the treatment of patients carrying dual activation of ALK and acquired mutations in these genes. On the other hand, secondary ALK activating mutations could be overcome by next generation ALK inhibitors; for example, *EML4-ALK* L1196M and G1269A activity could be effectively inhibited by several next-generation ALK-TKIs, including ceritinib, alectinib, and lorlatinib.<sup>21–23</sup> In this patient cohort, next-generation ALK-TKIs may benefit patients detected with secondary ALK activating mutations in their post-treatment samples.

Despite these results, there are some limitations to this study. First of all, this retrospective study was limited to patients with available samples for NGS. As such, our results need to be validated in a larger cohort. Secondly, the small sample size and targeted NGS may not adequately capture the full scope of resistance mechanisms.

In summary, we found heterogeneous resistance mechanisms in this patient cohort, including ALK activating

mutations, activation of bypass and downstream pathways, and DMMR deficiency, indicating diverse resistance mechanisms to crizotinib.

## Acknowledgments

We are grateful to the patients and their families for their support of this study. We also thank Pfizer Medical Affairs for scientific comments. This study was funded by: the National Key Technology R&D Program of the Ministry of Science and Technology of China: Prevention and Control of Major Non-communicable Diseases (Grant No. 2016YFC1303300); the Project of the National Natural Science Funding of China (Grant No. 81472207); National Key R&D Program of China (Grant No.2016YFC1303800); the Special Fund of Public Interest by the National Health and Family Control Committee (Grant No. 201402031); and the Guangdong Provincial Key Laboratory of Lung Cancer Translational Medicine (Grant No. 2012A061400006/2017B030314120).

## Disclosure

Yang W. Shao, Xue Wu, and Yan Ding are shareholders or employees of Geneseeq Technology Inc. Mei Wang, Jian-Gang Fu, and Zhenfan Yang are employees of Dizal (Jiangsu) Pharmaceutical Co. Ltd. Xian Zhang is an employee of Nanjing Geneseeq Technology Inc. Dr. Yi-Long Wu received lecture fees from Eli Lilly, Roche, Astra-Zeneca, Pfizer, and Sanofi.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** Flow chart of study enrollment process.

**Figure S2.** Structural modeling of ALK kinase domain carrying E1303K mutation after crizotinib treatment.

**Table S1.** Sample types and gene panels used for targeted next-generation sequencing (NGS).

**Table S2.** Clinicopathologic patient characteristics.

**Table S3.** ALK variant distribution according to next-generation sequencing (NGS).

**Table S4.** List of genetic alterations detected in this study.