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Concomitant resistance mechanisms to multiple tyrosine kinase inhibitors in *ALK*-positive non-small cell lung cancer

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ABSTRACT

Objectives: ALK tyrosine kinase inhibitors (TKIs), including crizotinib and several next generation TKIs, have demonstrated beneficial clinical outcomes in *ALK*-positive non-small cell lung cancer (NSCLC). However, resistance mechanisms following multiple TKI treatments in *ALK*-positive NSCLC are not fully elucidated.

Materials and methods: Mutation profiles of 422 cancer-relevant genes in 52 patients with post-TKI biopsy samples were analyzed using next-generation sequencing (NGS), and compared between patients receiving crizotinib alone (n = 35) and multi-TKIs (n = 17).

Results: *EML4-ALK* variant 3 is the most frequent *ALK* variants in this cohort, followed by *EML4-ALK* variant 1. Half of the patients harbored *ALK* activating mutations upon progression on crizotinib treatment. After multi-TKIs treatment, 59% of the cases developed resistant *ALK* mutations, and concomitant *ALK* activating mutations were more commonly observed in this cohort ($P = 0.031$). Specifically, *ALK* G1269 A, L1196 M, and C1156Y substitutions were more common in crizotinib-alone samples, while *ALK* G1202R was significantly more enriched post-multi-TKIs ($P = 0.009$). Activated bypass signaling tended to be more prevalent in patients post-multi-TKIs. Furthermore, dual activation of ALK and bypass signaling was more frequently found in the multi-TKIs group (5/17, 29%) in contrast to crizotinib-alone (2/35, 6%) ($P = 0.031$). Additionally, concurrent *TP53* mutation demonstrated significantly shorter progression-free survival (PFS) compared with *TP53* wildtype in crizotinib-alone group (median PFS: 8 vs 13 months, Hazard Ratio = 1.494, $P = 0.019$).

Conclusion: Concurrent *ALK* activating mutations and/or upregulated bypass signaling are more enriched in patients undergoing multiple ALK TKI treatments compared to crizotinib alone. Concomitant *TP53* mutation correlated to unfavorable survival when receiving a single TKI crizotinib.

1. Introduction

Anaplastic lymphoma kinase (*ALK*) gene arrangements are present in ~5% non-small cell lung cancer (NSCLC) [1]. The most common *ALK* rearrangement occurs due to an inversion within the short arm of chromosome 2 between *ALK* and the echinoderm microtubule-associated protein-like 4 (*EML4*) [2]. The breakpoint is mainly located upstream of the exon 20 of ALK, retaining its full tyrosine kinase domain in the resultant fusion protein, whereas rearrangements occur at several sites of *EML4*, giving rise to different *EML4-ALK* variants. Common *EML4-ALK* variants include variant 1 (v1, E13:A20), variant 2 (v2, E20:A20), and variant 3 (v3, E6:A20) [3]. Other rare fusion partners of *ALK* have also been reported in NSCLC, including kinesin family member 5B (*KIF5B*), TRK-fused gene (*TFG*), and kinesin light chain 1 (*KLC1*) [4–6].

ALK tyrosine kinase inhibitors (TKIs) has dramatically improved the clinical outcome of *ALK*-positive NSCLC patients. Crizotinib was the first ALK TKI approved for treating *ALK*-positive NSCLCs based on a

Abbreviations: ALK, anaplastic lymphoma kinase; CI, confidence interval; FISH, florescent in-situ hybridization; HR, hazard ratio; IHC, immunohistochemistry; KIF5B, kinesin family member 5B; KLC1, kinesin light chain 1; NGS, next-generation sequencing; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; TFG, TRK-fused gene; TKI, tyrosine kinase inhibitor

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significant improvement in overall response rate (ORR), progression-free survival (PFS), and quality-of-life [7,8]. However, patients who initially respond to crizotinib inevitably develop resistance due to over-activation of the ALK signaling such as *ALK* amplification and secondary activating mutations, or up-regulation of bypass signaling [9]. Several next generation ALK TKIs, including ceritinib, brigatinib, alectinib, and lorlatinib, have demonstrated favorable response to overcome crizotinib resistance in NSCLC, but resistance subsequently arise [10,11]. Mechanisms underlying multiple TKI resistance have not yet been fully elucidated.

In this study, we conducted comprehensive genomic profiling of the post-TKI treatment tumor biopsy samples from 52 *ALK*-positive patients by next-generation sequencing (NGS), and evaluated their resistance mechanisms to ALK TKI treatments when receiving crizotinib alone or subsequently multiple lines of TKIs.

2. Materials and methods

2.1. Patients

Post-TKI treatment samples of 52 Chinese patients diagnosed with *ALK*-positive NSCLCs from January 2011 to December 2017 at Shanghai Chest hospital were collected for mutation profiling of resistance mechanisms to ALK TKIs. Patients were separated into two cohorts, 35 of whom received crizotinib treatment alone, while the remainder was given multiple lines of TKI treatments followed by crizotinib, including ceritinib, brigatinib, alectinib and lorlatinib. Disease progression was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1) [12], and PFS was calculated from the date of TKI administration until progression was developed. Written consent was collected from each patient according to the Ethics Committee of the Shanghai Chest Hospital, Shanghai, China.

2.2. Next generation sequencing

Post-TKI treatment samples of 52 patients, including formalin-fixed paraffin-embedded (FFPE) or fresh tumor samples and matched plasma samples (if available), were subject to DNA extraction and comprehensive mutation profiling by targeted next-generation sequencing (NGS) with a panel of 422 cancer-relevant genes and selected introns of 19 genes frequently rearranged in solid tumors (Table S1) (GeneseeqOne, Nanjing Geneseeq Technology Inc., China), including the whole exons and intron 19 of *ALK* gene. Sequencing was performed on the Illumina HiSeq4000 platform followed by data analysis as previously described [13].

2.3. Statistical analysis

Categorical variables were compared between mutation carriers and non-carriers using the Fisher's exact test. The PFS of different cohorts were compared using the Mann-Whitney test. The PFS probabilities were analyzed using the Kaplan-Meier method where differences were calculated with Gehan-Breslow-Wilcoxon method and hazard ratio (HR) was calculated using log-rank method in Graphpad (Prism 6). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patients characteristics

Baseline characteristics of this cohort of patients were summarized in Table 1 with details outlined in Table S2. The median age of this cohort is 50 years old with no bias in the genders and mainly stage IV adenocarcinoma at diagnosis. All patients were tested ALK-positive by FISH or IHC, and received crizotinib as first-line TKI treatment, while 17 patients underwent one or two next generation ALK TKI therapies (Multi-TKIs group), including alectinib, ceritinib, brigatinib, and lorlatinib, after disease progressed on crizotinib (Fig. 1 and Table S2). The progression-free survival (PFS) of crizotinib in patients who received multiple TKIs was slightly shorter, but not significantly different from that of patients on crizotinib alone (PFS median: 8 vs 11 months, $P = 0.172$). Neither was the overall PFS of patients who received multi-TKIs significantly different from that of patients who were treated with crizotinib alone (median: 16 vs 11 months, $P = 0.079$).

Table 1
Baseline clinical characteristics of patients in this cohort.

Characteristic		Number (%)
No. of patients		52
Median age, years (range)		50 (27-75)
Sex		
Male		29 (56)
Female		23 (44)
Pathology		
Adenocarcinoma		51 (98)
Squamous cell carcinoma		1 (2)
Stage at Diagnosis		
I		0
II		0
III		3 (6)
IV		49 (94)
Brain metastasis at diagnosis		
Yes		5 (10)
No		47 (90)
Line of treatment of crizotinib		
One		21 (40)
Two		17 (33)
Three or higher		14 (27)
Number of ALK TKIs		
Mono-TKI (crizotinib)		35 (67)
Multi-TKI	Two TKIs	13 (25)
	Three TKIs	4 (8)
ALK TKI treatments		
Crizotinib		52 (100)
Brigatinib		8 (15)
Ceritinib		8 (15)
Alectinib		3 (6)
Lorlatinib		2 (4)

All post-TKI samples from these patients were tested positive for *ALK* fusions by targeted NGS (Fig. 1 and Table S2). *EML4-ALK* v3 and v1 were the two most common variants of *ALK* fusion, accounting for 52% (27/52) and 27% (14/52) of the cohort, respectively (Figure S1). Other *EML4-ALK* variants, include v2 (E20:A20), v5′ (E18:A20), v7 (E14:A20), unnamed variants including E5:A20 and E21:A20, were detected in approximately 15% (8/52) of patients, and the remaining 3 cases (6%) were non-*EML4-ALK* rearrangements which involve the rare fusion partners including *STRN*, *HIP1* and *MEMO1* (Figure S1 and Table S2). The PFS of crizotinib treatment in these patients were not associated with different ALK fusion variants in this cohort.

3.2. Concomitant resistance mutations are more common following multi-TKI treatments

Mutation profiling of post-TKI samples was performed by NGS targeting 422 cancer-relevant genes (Table S2 and Figure S2). As shown in Fig. 1, *ALK* activating mutations were detected in a total of 27 patients in 59% (10/17) of the Multi-TKIs group and 49% (17/35) of the crizotinib-alone group ($P = 0.56$). Of note, *ALK* G1202R substitution was more commonly observed in the Multi-TKIs subset (41%, 7/17) than crizotinib-alone (9%, 3/35) ($P = 0.009$), while *ALK* L1196 M, G1269 A and C1156Y tended to be more enriched in crizotinib-alone group (Fig. 1 and 2). In particular, *ALK* L1196 M represents the most common mutation with a frequency of 14% (5/35) following crizotinib single agent treatment, but was not identified in the Multi-TKIs cohort, consistent with a previous report that this mutation is sensitive to next

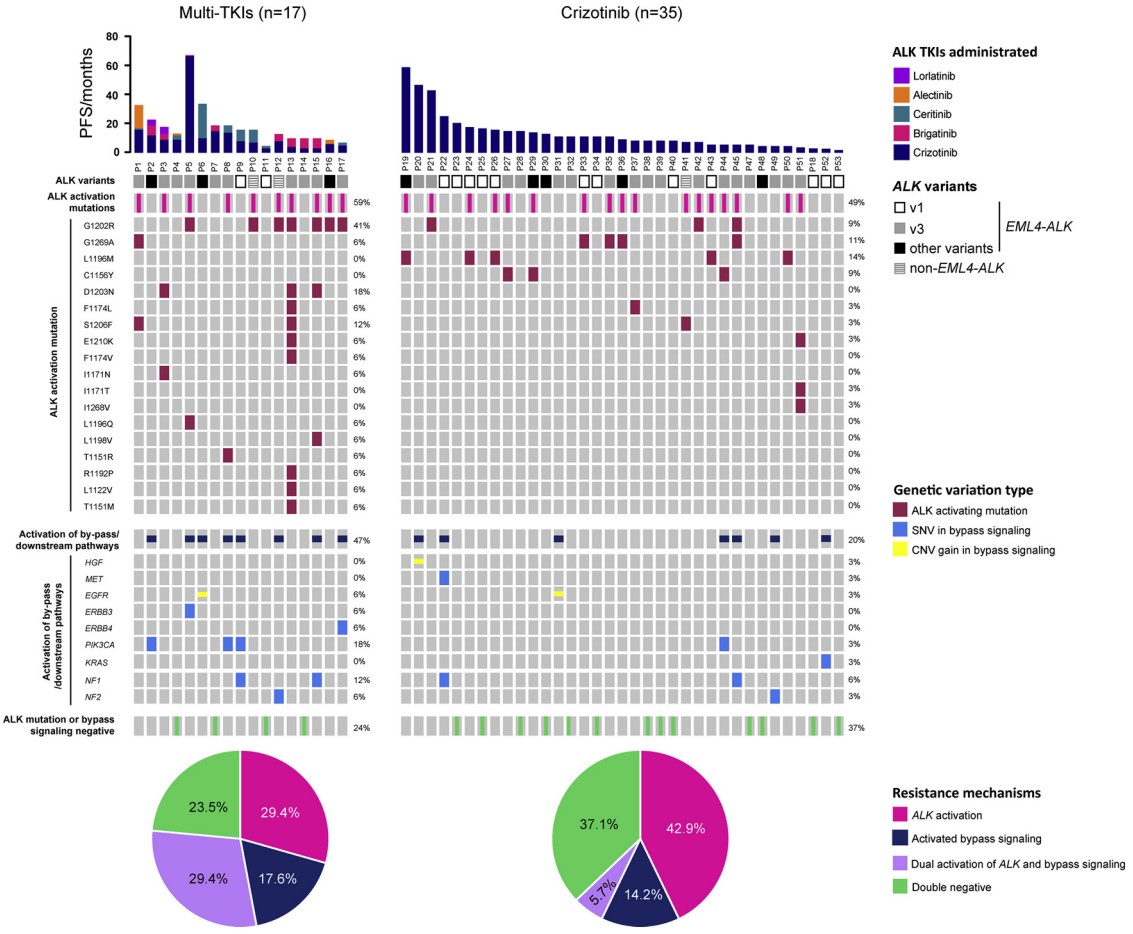

Fig. 1. *ALK* activating mutations and bypass signaling activation in the multi-TKI and crizotinib-alone groups. *ALK* variants are labeled under patient ID. PFS of each ALK TKI treatment is plotted above patient ID. Resistance mechanisms and genetic variation types are color coded as indicated. Mutations identified in either tissue samples (FFPE or fresh tumor) or plasma sample (if available) for each patient were included for the co-mutation plot.

generation ALK TKIs including alectinib, ceritinib, brigatinib, and lorlatinib [14]. Furthermore, patients with concomitant *ALK* activating mutations were more frequently seen in Multi-TKIs (29%, 5/17) comparing to the crizotinib-alone subset (6%, 2/35) (P = 0.031, Fig. 1 and Fig. 2C).

Aside from *ALK* activating mutations, we also observed the activation of bypass signaling, including amplification of *HGF* and *EGFR*, and somatic mutations in *MET*, *ERBB3*, *ERBB4*, *PIK3CA*, *KRAS*, *NF1* and *NF2*, in a total of 15 patients, with 8 (47%) in Multi-TKIs and 7 (14%) in crizotinib-alone (P = 0.056, Fig. 1 and Figure S2). Importantly, dual activation of both ALK via *ALK* activating mutation and bypass signaling were seen in a subset of 7 patients (Fig. 1), and of note, this dual activation was significantly more enriched in patients who underwent multiple lines of TKI treatment in contrast to crizotinib-alone (29.4% vs. 5.7%, P = 0.031, Fig. 1). However, 23% (4/17) of patients in multiple TKIs group and 37% (13/35) of crizotinib-alone treated patients were neither *ALK* activating mutation nor known bypass signaling activation.

3.3. TP53 correlates to poor PFS in patients receiving crizotinib alone

TP53 represents the second most frequently mutated gene affecting approximately 56% (29/52) of patients with 21 patients in crizotinib-alone and the remaining cases (n = 8) in Multi-TKIs (Figure S2 and Table S2). Patients carrying mutated *TP53* had significantly shorter PFS when comparing to non-carriers in crizotinib-alone cohort (median PFS: 8 months vs 13 months, HR = 1.494 [95% CI, 0.8290–3.120], P = 0.0189, Fig. 3A), while the presence of *ALK* activating mutation was not significantly associated with PFS in either $TP53^{m+}$ or $TP53^{wt}$ cohorts (Fig. 3B). Although we did not observe significant difference in PFS between *EML4-ALK* v3 and non-v3 in the entire cohort or in patients with mutated *TP53* (Figure S3 A), v3 patients tended to be worse in PFS than non-v3 patients in patients without a concurrent *TP53* mutation (P = 0.1330, Figure S3B). Together, these results suggest that *TP53* serves as a negative prognostic factor in *ALK*-positive NSCLCs under crizotinib treatment.

4. Discussion

Herein, we report that *EML4-ALK* v3 and v1 were the most common *ALK* variants identified in this cohort of 52 Chinese *ALK* fusion NSCLC patients. Secondary mutations of the *ALK* gene were identified in ~49% of patients who were treated with crizotinib alone. The mutation rate is higher than the previous report by Katayama et al that ~27% of ALK fusion NSCLC patients were positive of *ALK* mutations post crizotinib [9], but is comparable to Doebele et al that 44% (4/9) *ALK* fusion positive patients were identified with point mutations in ALK kinase domain following crizotinib [15]. Comparing to crizotinib alone, a higher frequency of *ALK* activating mutations (59%) was seen for patients who underwent multiple lines of ALK TKI treatments, although the overall PFS of this subset of patients was not significantly different from that of patients treated with crizotinib alone. Concomitant *ALK* activating mutations were found to be more common following multi-TKI treatments in contrast to a single agent crizotinib. Our finding is in line with a recent report showing that a sequential TKI treatment could select for concomitant *ALK* mutations that confer high-level resistance

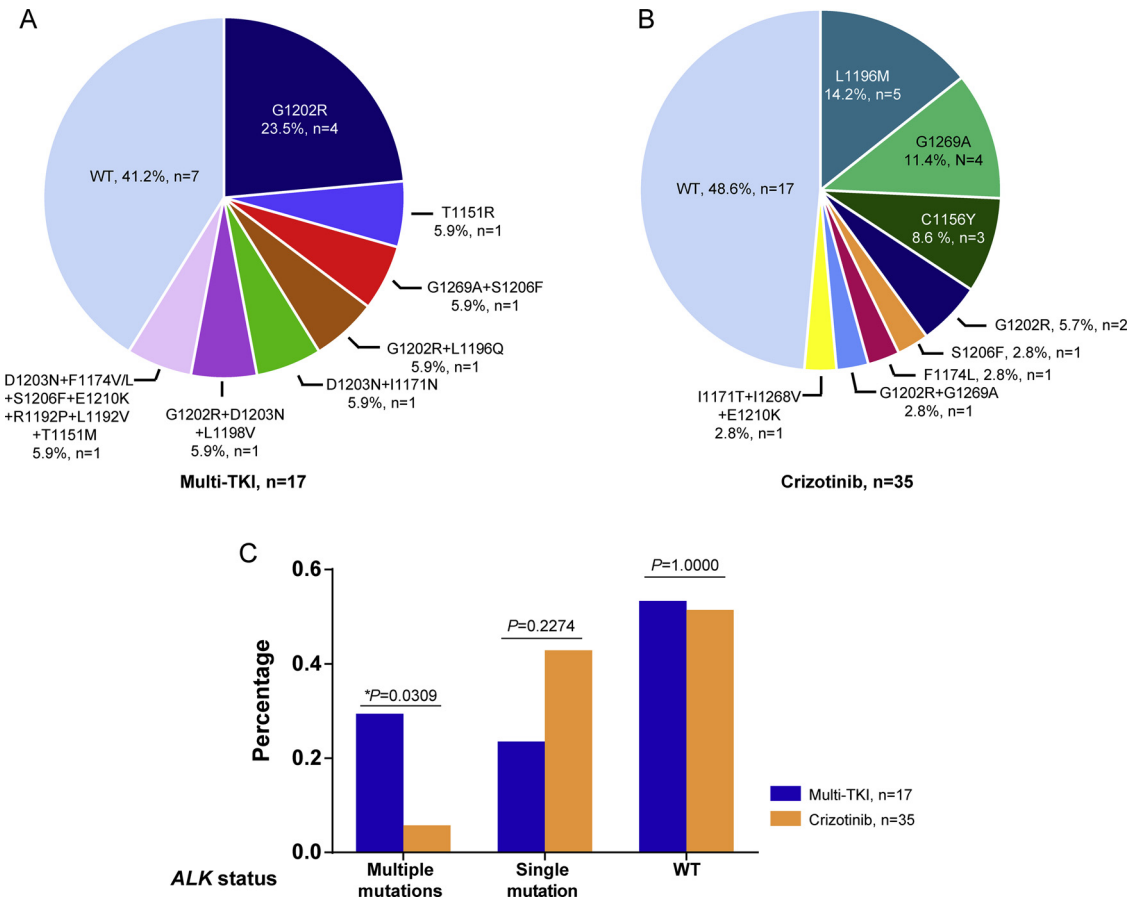

Fig. 2. Mechanisms of resistance to ALK TKIs in the multi-TKI and crizotinib-alone groups. (A) Summary of *ALK* activating mutations detected in the multi-TKI group after the development of resistance to the last TKI administrated. (B) Summary of *ALK* activating mutations detected in the crizotinib group after the development of resistance to crizotinib. (C) Comparison of the number of *ALK* activating mutations detected in each patient between multi-TKI and crizotinib groups. Multiple mutations: more than 1 *ALK* activating mutation was detected in 1 sample; single mutation: 1 mutation was detected in 1 sample. *P* value was calculated according to Fisher exact test.

to ALK-targeted therapies [14]. Furthermore, activation of bypass signaling pathway was observed in ~20% of patients on crizotinib alone, which is comparable to a previous finding reported by Doebele et al [15]. However, a relatively higher rate (47%) of bypass signaling activation was seen for patients treated with multi-TKIs, and a dual activation of *ALK* and bypass signaling was significantly more enriched in these patients, further supporting that the sequential TKI treatment may enrich for concurrent *ALK* mutations and bypass signaling activation upon disease progression. This is also consistent with the finding that *ALK* resistance mutations are more common after treatment with second-generation ALK inhibitors by Gainor et al. [11] as they observed that 54% of ceritinib-resistant specimens harboured *ALK* resistance mutations.

In particular, a total of 6 patients received brigatinib treatment after progressed on crizotinib with PFS of 5–7 month, except for P5 who continued to progress after the administration of brigatinib with ALK G1202R/L1196Q mutations despite that he achieved a PFS of 67 months to crizotinib (Table S2). Patients P12 and P15 were positive for both *ALK* activating mutation G1202R and bypass signaling activation through deactivating NF family genes post treatment. Patient P13 harbored numerous *ALK* point mutations, including G1202R, D1203 N, S1205 F, E1210 K, F1174 L/V, R1192 P, L1127 V, and T1151 M, which may collectively contribute to the drug resistance. The remaining two patients (P7 and P14) were negative for either *ALK* secondary mutations or bypass signaling activation. Two patients (P2 and P3) received lorlatinib treatment post progression on brigatinib, and consistent to the previous findings, G1202R was not observed in these two patients. P2 harboured a *PIK3CA* activating mutation E542 K, while P3 has *ALK* D1203 N and I1171 N mutations. Seven patients received ceritinib with various potential resistant mechanisms, including *ALK* mutations (G1202R and T1151R), *EGFR* amplification, and *PIK3CA* mutation. Future studies of larger cohort size will be required for investigating resistance mechanisms to second generation ALK TKIs.

We also report that mutated *TP53* significantly correlated to poor PFS in *ALK*-positive NSCLC patients who received crizotinib alone. It has previously been reported by Li et al [16] that *TP53* mutation showed a correlation to unfavorable crizotinib PFS in sixty *ALK*-positive NSCLC patients although with a borderline *P* value (0.068). These findings together support that *TP53* represents a poor prognostic factor in NSCLCs with targetable mutations including *EGFR, ALK,* and *ROS1* [17]. In addition, previous reports argued that different *EML4-ALK* variants might give rise to distinct clinical outcomes in response to ALK TKIs [18,19]. The longer *EML4-ALK* variants (v1 and v2) retained the full truncated tandem atypical β-propeller (TAPE) domain of *EML4* that increases the instability of the fusion protein, while the shorter *EML4-ALK* variant (such as v3) is more stable due to the lack of TAPE domain [20]. However, we did not observe significant difference of clinical benefits between *ALK* variants in this study likely due to the small cohort size.

In summary, this study demonstrates that concurrent *ALK* activation mutations, or dual activation of ALK and bypass signaling, were more enriched in patients receiving multiple lines of TKI treatments when comparing to a single agent such as crizotinib. In addition, co-existing *TP53* mutation correlated to unfavorable survival in *ALK*-positive

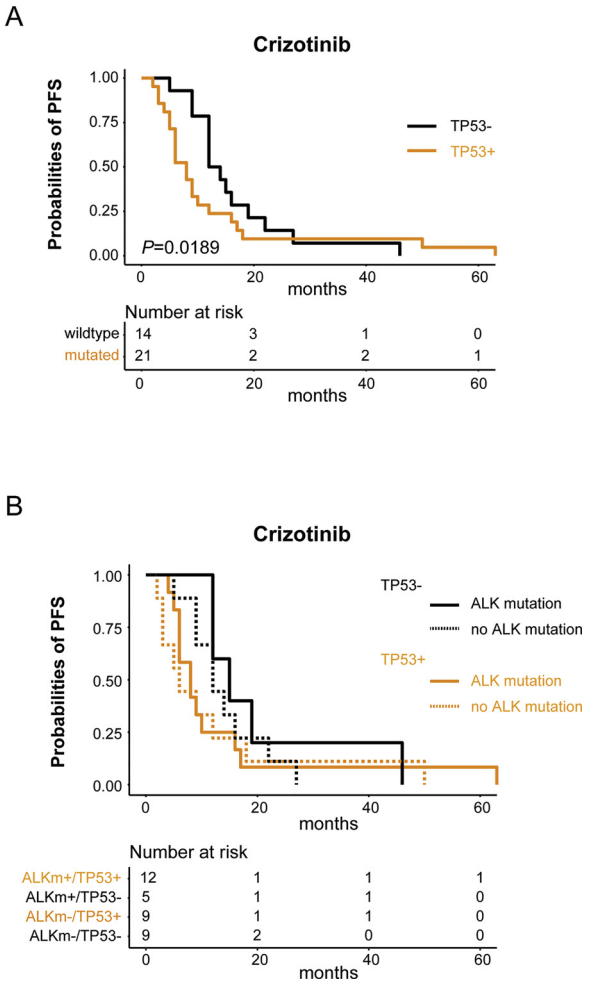

Fig. 3. Concurrent *TP53* mutations correlate to unfavorable PFS in patients treated with crizotinib alone. (A) Kaplan-Meier curves comparing PFS of with or without co-existing *TP53* mutations. (B) Kaplan-Meier curves comparing PFS by the presence of co-existing *TP53* mutations and *ALK* activating mutations. *P*-value is estimated using the Gehan-Breslow-Wilcoxon test in Graphpad Prism 6.

NSCLC patients under crizotinib.

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Conflicts of interest

Qiuxiang Ou, Xue Wu, Yan Ding and Yang W. Shao are the shareholders or employees of Geneseeq Technology Inc. Canada; Hairong Bao is the employee of Nanjing Geneseeq Technology Inc. The remaining authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2018.11.024.

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