Peer Review File

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Reviewer A

This is a very interesting report, but suffers from a major methodologic limitation: the results are possibly confounded by the fact that the 2G ALK inhibitors were used instead of crizotinib in almost 50% (44%) of patients with multiple ALK fusions vs. only approximately 25% (23%) of patients with single ALK fusions (lines 199-203). This can by itself explain why patients with multiple ALK fusions appeared to have a longer PFS in this study. Along these lines, the median PFS of patients with multiple ALK fusions was consistent with that of 2G TKI (26.9 mo), while that of patients with single fusions was typical of crizotinib (11.2 months, lines 223-225). The only way (far from perfect) to address this would have been a multivariate analysis, but the ALK TKI generation was not included as a parameter in the multivariate analysis of Table S2 (even though it was included in univariate analyses). One reason to address the concern of potential confounding before publication is that previous studies (like ref. 29) have suggested that more complex translocation events might be a marker of higher genetic instability and associated with worse prognosis (which contrasts the better prognosis reported in case of multiple fusions in the manuscript under review)

Reply: Thank you underlining this deficiency. We have revised this section and modified according to the comments you suggested. We added the ALK-TKI generation as a parameter in the multivariate Cox regression (see Page 8, Line 249-252), and we found that multiple ALK fusions tended to predict better prognosis though there was no statistical difference. Then we conducted a subgroup analysis in different ALK-TKI generation, and found than both in first and second generation ALK-TKI, patients harboring multiple ALK fusions had favorable PFS and OS, especially in first generation group, patients harboring multiple ALK fusions had a significant difference in PFS (11.0 months vs. 21.1 months, *P*=0.049) (see Page 8, Line 252-258). Moreover, for the potential confounding of genetic instability, we explained it in the Discussion section (see Page 10, Line 345-348).

Change in the text: Page8, Line 249-252. Page 8, Line 252-258. Page 10, Line 345-348.

Reviewer B

Authors showed ALK-TKI-treated lung cancer patients with multiple ALK fusions had longer PFS than those with a single EML4-ALK fusions (26.9 vs. 11.2 months, P=0.009). Multivariate Cox regression analysis confirmed that harboring multiple ALK fusions was an independent predictor of better PFS for ALK-positive NSCLC (P=0.019).

Authors described the association between multiple ALK fusions and improved PFS in NSCLC patients undergoing treatment with ALK-TKIs. I have two comments although this manuscript had first comprehensive analysis concerning multiple ALK-fusions.

Authors analyzed PFS and OS data from 56 ALK-positive (single 44 and multiple 12) patients treated with ALK-TKI at their institution, and 6 patients with multiple fusion in the literature

search. From literature search, the analytic methods using NGS are variable and there are many ways to collect clinical data such as OS and PFS. How about adding just the data of their own facility although I understand patients with multiple fusions are only 12?

I have no idea why multiple ALK fusions had longer PFS than those with a single EML4-ALK fusions. Are there preclinical data suggesting it?

Reply: Thank you for your comments on the manuscript. First, we added data of 56 patients in our hospital to avoid the bias from literature search in addition (see Page 8, Line 259-263). However, for the second comment of why multiple ALK fusions had longer PFS than those with a single EML4-ALK fusions, there is no clear understanding on it, and the mechanism of prolonged survival of multiple ALK fusion is still unknow, and we conducted literature search of PubMed and Embase for preclinical data, we found no preclinical data explaining this survival benefit. However, through literature retrieval, we analyzed the possible reasons for this survival benefit (see Page 10, Line339-342, Line 349-354), and we will continue to explore reasons in the future study.

Change in the text: Page 8, Line 259-263. Page 10, Line339-342, Line 349-354.

Reviewer C

The paper describes the outcome of NSCLC patients who harbor multiple ALK aberrations in their tumors. A total of 125 patients with advanced ALK-positive NSCLC were entered into this retrospective study. Multiple LAK aberrations were detected in 21% of patients. These patients had longer progression-free survival compared to the other patients. Overall survival was not different but this lack of statistical difference might have been affected by the low number of patients. Overall, the findings of this retrospective study are of clinical interest.

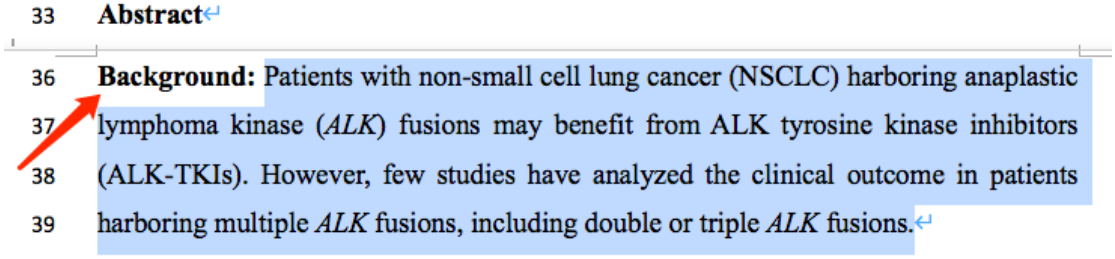
Reply A: Thank you for your time to review this manuscript, and thank you for your comments. We acknowledge that the number of patients in our study was a little small, in our study, we found that multiple *ALK* fusions have the potential to predict superior clinical outcome in patient with NSCLC, but studies on the clinical outcome of a larger cohort of patients with multiple *ALK* fusions are warranted.

Change in the text: There is no change in the text.

Reviewer D

1. **Abstract**

a. Please extend the content of the Background. This paragraph should contain 'study background' and 'study objective'.



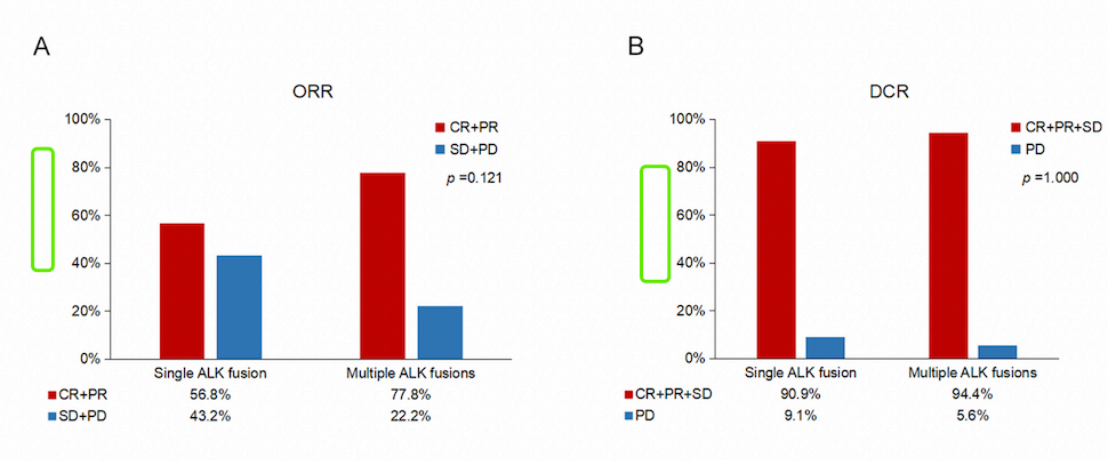
b. This keyword is not appeared in abstract or the main text. Please revise.

60 **Keywords:** Multiple anaplastic lymphoma kinase fusions (multiple *ALK* fusions);
61 *ALK*-rearranged lung cancer; prognosis; single *ALK* fusion; distribution of *ALK* fusions
62

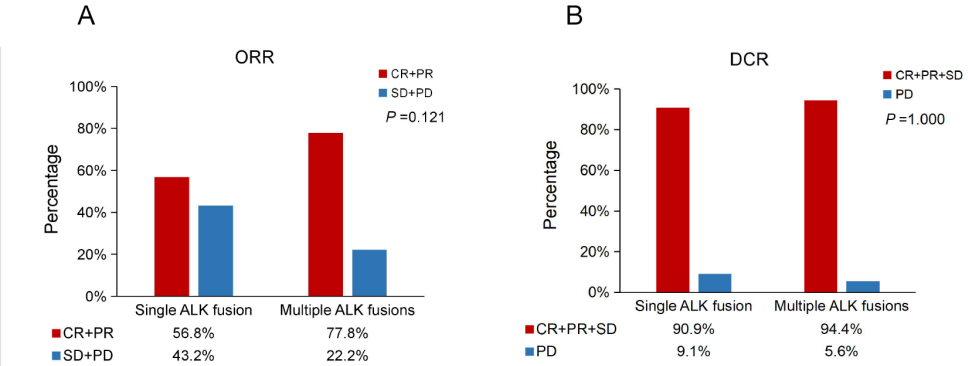
Reply 5: We have extended the content of the background, and corrected the keyword.
Changes in the text: Page 1, Line 40-42; Page 2, Line 64.

2. Figure 2

Please add description for the Y-axis.



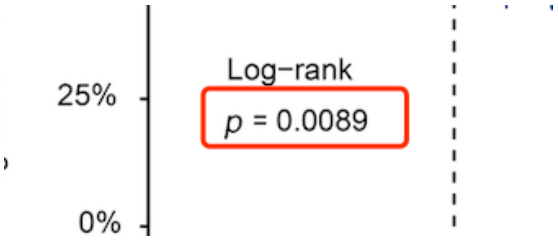
Reply 6: We added the description for the Y-axis of figure 2.
Changes in the text: Page 18, Line 598 (figure-2-revised).



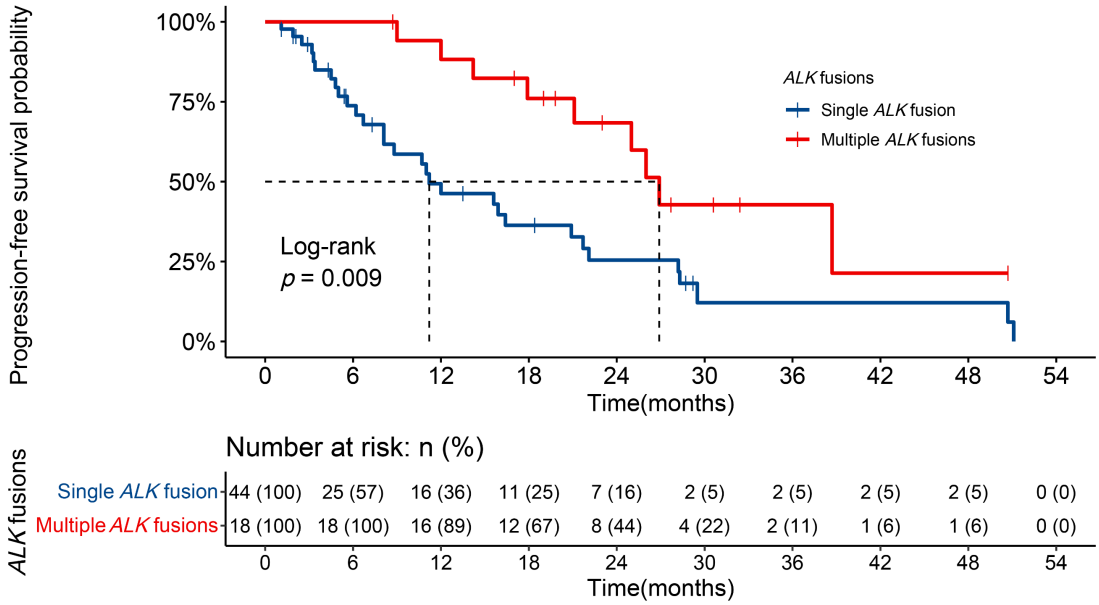
3. Figure 3

Please check which P value is correct.

235 increased in the patients with multiple *ALK* fusions (26.9 months) compared to those
236 with a single *ALK* fusion (11.2 months, P=0.009; *Figure 3*). For OS, as shown in *Figure*



Reply 7: The P value was 0.009, and we have corrected the figure 3.
Changes in the text: Page 19, Line 609 (figure-3-revised).



4. Figures should be cited consecutively in order in the main text. However, figure S3 was cited before figure S2, which is not allowed. Please revise. You can rename figure S3 as figure S2, and rename the original figure S2 as figure S3.

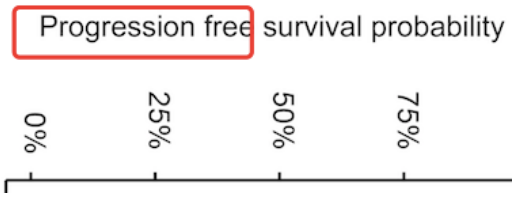
259 than those harboring single ALK fusion, although there were no statistically signific
260 differences in patients who received second-generation ALK-TKIs (*Figure S3*).
261 To reduce the data bias, we further analyzed the 56 patients came from our hospital

285 26.9 months, P=0.17) (Figure S2A), and there was also no significant difference in OS
286 between these two groups (P=0.15) (Figure S2B). Moreover, for patients without brain
287 metastases, we found a significantly better PFS in the multiple *ALK* fusion group (26.0
288 *vs.* 15.6 months, P=0.028) (Figure S2C), but no significant difference in OS was
289 reported between these two groups (P=0.763) (Figure S2D).

Reply 8: We have renamed figure S3 as figure S2, and rename the original figure S2 as figureS3.
Changes in the text: Page 23, Line 645 (figure-S2-revised); Page 24, Line 670 (figure-S3-revised).

5. **Figure 3, Figure S2, Figure S3**

Please revise “Progression free” to “Progression-free”.

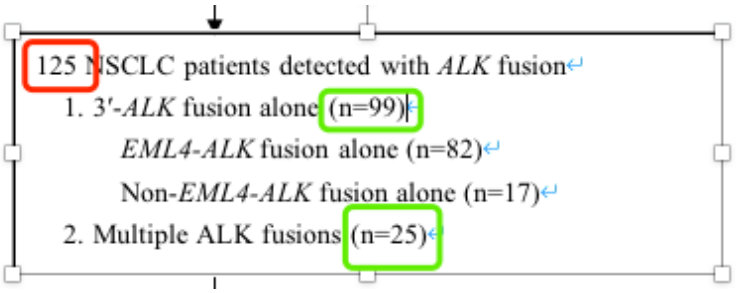


Reply 9: We have revised.

Changes in the text: Page 19, Line 609 (figure-3-revised); Page 23, Line 645 (figure-S2-revised); Page 24, Line 670 (figure-S3-revised).

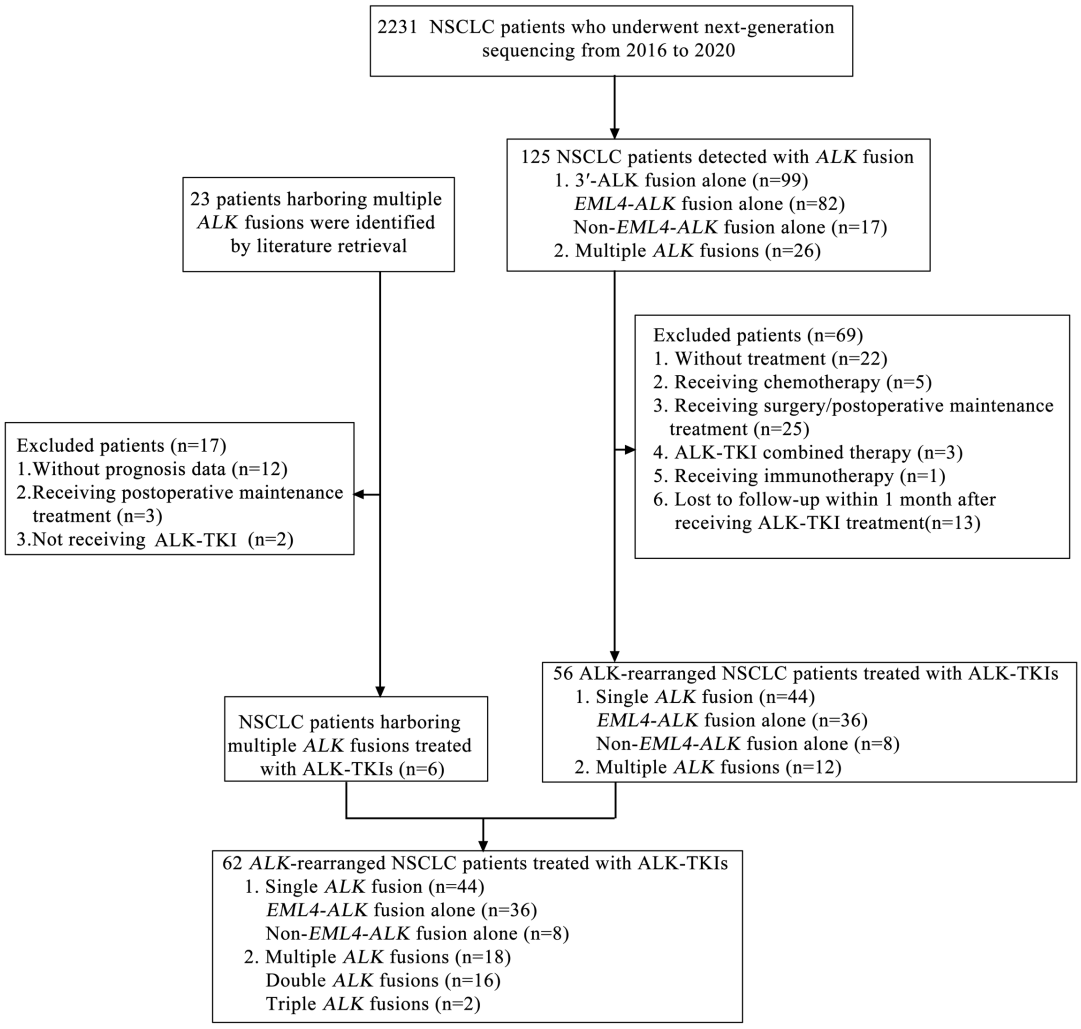
6. Figure S1

99 plus 25 is 124, not 125. Please double check the accuracy of data.



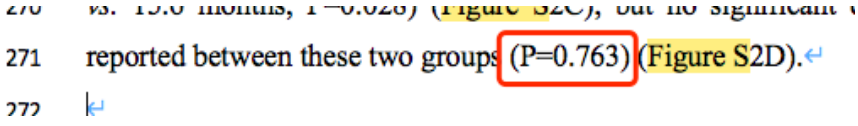
Reply 10: We have checked and corrected the figure S1.

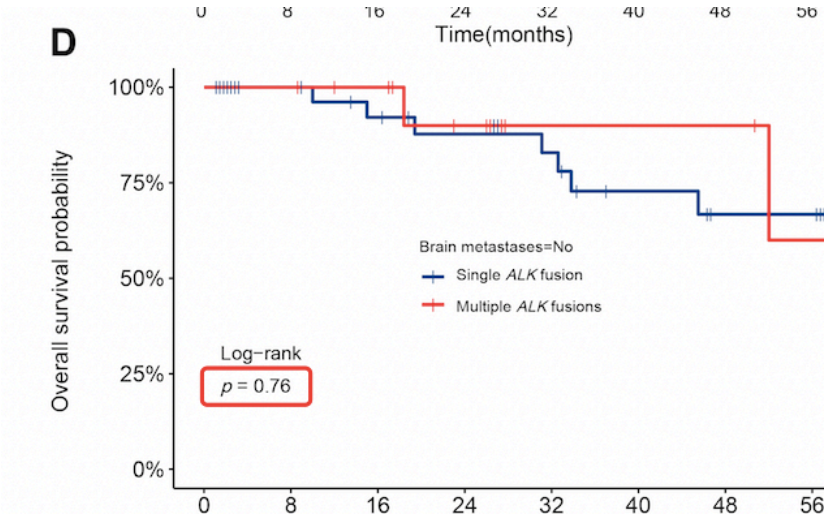
Changes in the text: Page 22, Line 638 (figure-S1-revised), and the figure-S1-word-revised.



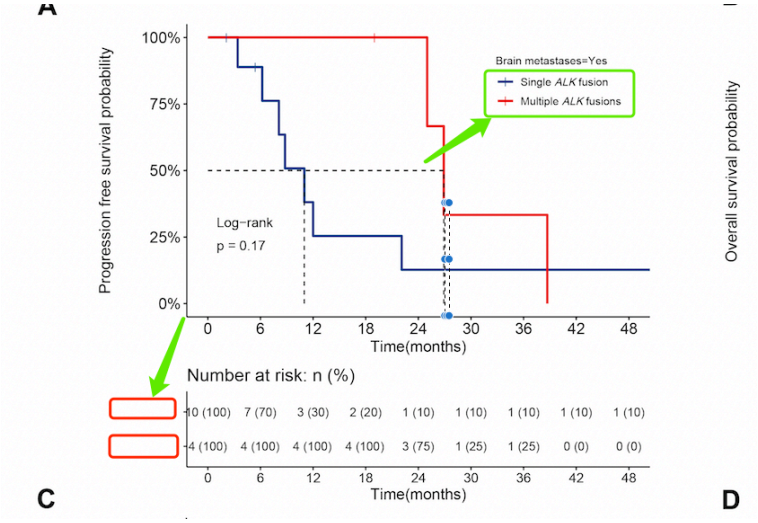
7. Figure S2

a. Please check which P value is correct.



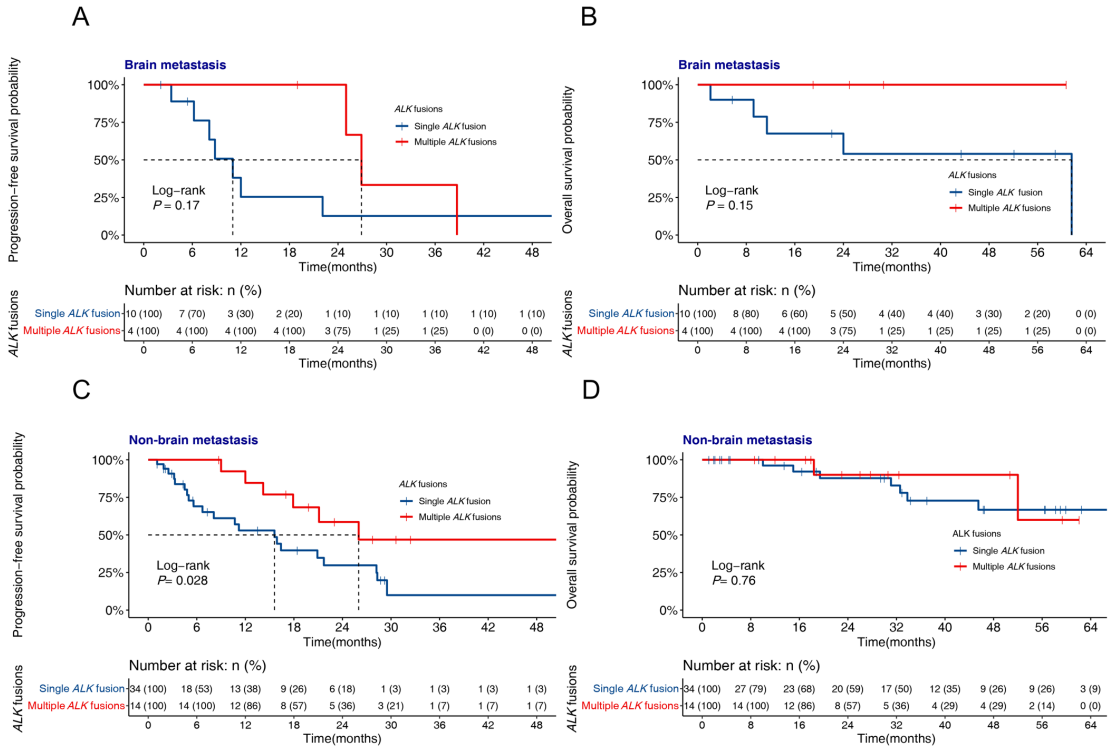


b. the legend pointed in green box should also be indicated in the red box. Please revise the whole figure S2.



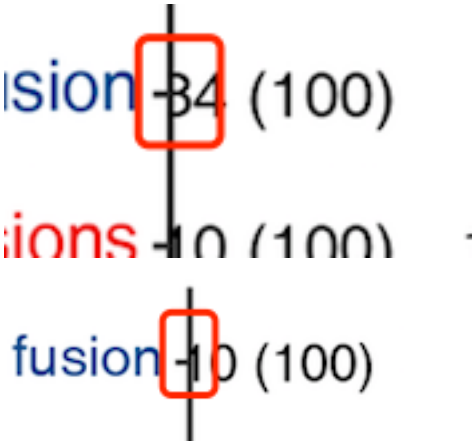
Reply 11: The P value was 0.76, we have checked and corrected it; We have revised the whole figure S2.

Changes in the text: Page 9, Line 293; Page 23, Line 645 (figure-S2-revised).



8. Figure S3A, B, C, D

Some numbers are overlapped. Please revise.



Reply 12: We have revised.

Changes in the text: Page 24, Line 670 (figure-S3-revised).

9. Table S3

It seems that “Median (range)” should be “Median [range]”

643 **Table S3** Baseline characteristics of the 56 patients with *ALK*-rearranged NSCLC who
644 received ALK-TKI therapy

Characteristic	All, n (%)	Single *ALK* fusion, n (%)	Multiple *ALK* fusions, n (%)	*P*
No. of patients	56	44 (78.6)	12 (21.4)	
Age, Median (range)	51.5 [42.0, 61.0]	51.0 [43.5, 64.3]	53.00 [41.8, 57.5]	0.667
Age, n (%)				

Reply 13: We have revised the round bracket into square bracket.
Changes in the text: Page 29, Table S3 (Table-S3-revised).

10. Table S4

(95% CI) data should be in round bracket. Please revise the whole table S4.

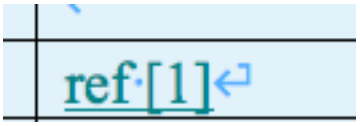
648 Table S4 Univariate and multivariate Cox regression analysis of 56 patients

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P	HR (95% CI)	P
Age	1.011 [0.984,1.039]	0.439		
Gender				

Reply 14: We have revised the whole table S4.
Changes in the text: Page 30-31, Table S4 (Table-S4-revised).

11. Table S2 and Table S4

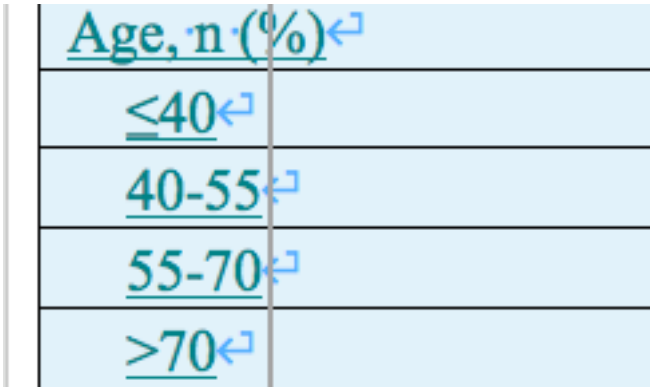
Does the pointed "[1]" has any meaning? If so, please supplement. If not, please remain "Ref" and delete "[1]". Please check the whole figure and revise.



Reply 1: There was no meaning of the point [1], and we have deleted [1] and remained "ref".
Changes in the text: Page 28, Table S2; Page 31, Table S4.

12. Table S3

1) The two groups "44-55" and "55-70" include 55 at the same time. Please check and revise.



2) Should the P value be filled in the pointed boxes? Please check and revise.

Characteristic	All, n (%)	Single *ALK* fusion, n (%)	Multiple *ALK* fusions, n (%)	*P*
No. of patients	56	44 (78.6)	12 (21.4)	
Age, Median[range]	51.5 [42.0, 61.0]	51.0 [43.5, 64.3]	53.00 [41.8, 57.5]	0.667
Age, n (%)				
≤40	10 (17.9)	8 (18.2)	2 (16.7)	0.999
40-55	23 (41.1)	18 (40.9)	5 (41.7)	
55-70	18 (32.1)	14 (31.8)	4 (33.3)	
>70	5 (8.9)	4 (9.1)	1 (8.3)	
Gender, n (%)				
Male	27 (48.2)	20 (45.5)	7 (58.3)	0.642
Female	29 (51.8)	24 (54.5)	5 (41.7)	
Smoking status, n (%)				
Never	40 (71.4)	31 (70.5)	9 (75.0)	0.648
Former/Current	13 (23.2)	10 (22.7)	3 (25.0)	
Unknown	3 (5.4)	3 (6.8)	0 (0.0)	
ECOG PS, n (%)				
0	15 (26.8)	11 (25.0)	4 (33.3)	0.521
1	30 (53.6)	23 (52.3)	7 (58.3)	
≥2	11 (19.6)	10 (22.7)	1 (8.3)	
Pathology, n (%)				
Squamous cell carcinoma	1 (1.8)	1 (2.3)	0 (0.0)	0.754
Adenocarcinoma	54 (96.4)	42 (95.5)	12 (100.0)	
Adenosquamous carcinoma	1 (1.8)	1 (2.3)	0 (0.0)	
Stage, n (%)				
III	12 (21.4)	10 (22.7)	2 (16.7)	0.507
IVA	20 (35.7)	14 (31.8)	6 (50.0)	

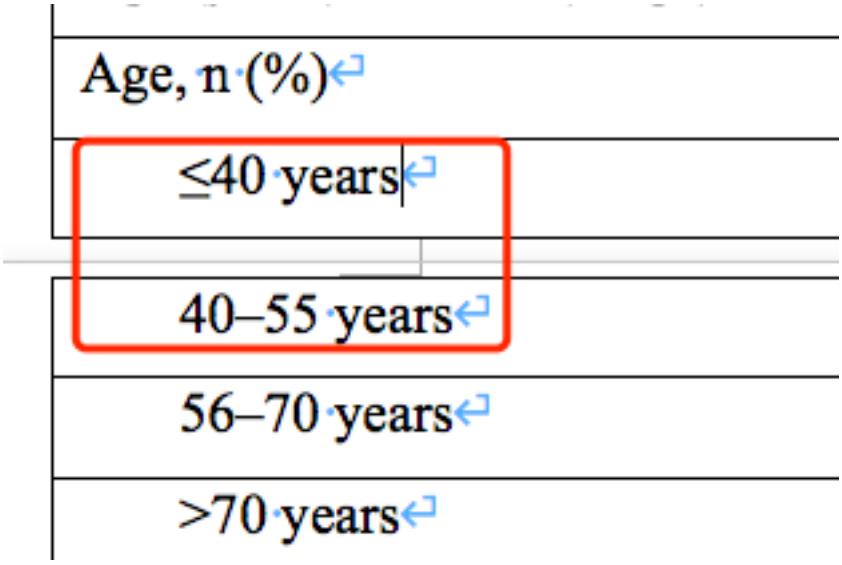
Reply 2:

1) We have checked the two group and changed it into “40-55” and “56-70”. The same error was found in Table 1 and we have revised it.
2) We have checked it and revised the boxes of P value.

Changes in the text: Page 24, Table 1; Page 29, Table S3.

13. Table 1 & Table S3

The two groups “≤40" and “40–55” include 40 at the same time. Please check and revise.



Reply1: We have checked the two groups and changed it into “⩽40” and “41-55”.

Changes in the text: Page 24, Table 1; Page 29, Table S3.