Peer Review File

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Reviewer A

Minor Comment

The authors might consider replacing “step 1 (or 2) of the trial” by “part 1 (or 2) of the trial”.

#Reply:

Thank you for this valuable suggestion. As recommended, we have replaced step 1 with phase 1, and step 2 with phase 2 throughout the manuscript and Supplementary Figure 1.

Reviewer B

General remarks

The authors could pay more attention to this reason why alectinib might not be the ideal drug to target RET.

#Reply:

Thank you for valuable comments. We revised the paper according to you suggestions.

Major remarks

Comment 1: How many patients had brain metastases? The authors should add this to the respective tables, as this is an important prognostic factor for NSCLC patients.

Reply 1:

Four patients in phase 1 and 4 patients in phase 2 with brain metastases at baseline determined by the central review board were enrolled in this study. We have added this information in Table 1.

Comment 2: The timelines of the study are unclear, as in line 126 it says: “As of January 2017…”, while in the Results section it says “Between February 2013 and January 2018” for the screening study and “Between March 8, 2016 …”. The authors should clarify this.

Reply 2:

We apologize for the typographical error. We conducted this study in January 2016 and have corrected the sentence on page 7, line 142. The screening study “LC-SCRUM-Japan” started from Feb 2013 is separate from this ALL-RET clinical trial.

Comment 3: Lines 294-295: the authors are comparing the measured Cmax concentrations to the IC50 values. However, they measured total concentrations of alectinib (i.e. free fraction plus fraction bound to plasma proteins). As only the free fraction can be pharmacologically active and alectinib is for > 99% bound to plasma proteins, this comparison could not be made in this way. Also, Cmin is probably a more relevant pharmacokinetic parameter in terms of efficacy, as target inhibition is needed during the complete dosing interval.

Reply 3:

Considering this comment, we have revised the sentences on page 14, lines 330-334 as follows:

"The free fraction of Cmin in a steady state of alectinib 450 mg BID (<4.8 ng/mL, equivalent to <10 nmol/L) treatment would be comparable to the half-maximum inhibitory concentration (IC50) of RET kinase (4.8 nmol/L) and much lower than that in the RET-fusion positive NSCLC cells (< 300 nmol/L) (20, 21). Therefore, it can be one of the reasons why alectinib showed limited activity against NSCLC with RET rearrangement."

Comment 4: Of the 119 patients in whom a RET-rearrangement was identified in the LC-SCRUM-Japan study, only 34 patients were enrolled in the study. What were the reasons that other patients were not enrolled? Was it because the study was not open for enrollment yet? Or were these patients included in other studies? Could selection bias have played a role here?

Reply 4:

Cases of RET-rearranged NSCLC are rarely encountered. To identify such patients, we initiated a prospective observational study named LC-SCRUM-Japan in Feb 2013 with a nationwide genomic screening in Japan, which is separate from this ALL-RET clinical trial. Hence, RET-rearranged NSCLC patients were first identified in the LC-SCRUM-Japan study. Following this, only patients who met the eligibility criteria for the ALL-RET study were enrolled, which was initiated in March 2016. The main reasons other patients were excluded include 1) ALL-RET study was not opened for patients before March 2016, and 2) the patients did not demonstrate progression of tumor after previous chemotherapy treatment. During the enrollment period for ALL-RET, other clinical trials examining RET-inhibitors were not initiated in Japan. We believe that ALL-RET has no selection bias.

Considering this comment, we changed the sentence (page 11, lines 246-249) as follows.

"Between February 2013 and January 2018, 4552 patients with advanced non-squamous NSCLC enrolled in the LC-SCRUM-Japan study were screened for RET rearrangements. Among them,

119 patients (2.6%) with RET-rearranged NSCLC were identified, and a total of 34 patients who met the eligibility criteria were enrolled in the present study."

Comment 5: In Table 3, the authors report Cmin for Day 1, but according to the sampling schedule, they did not collect a Cmin sample at this time point, also the ranges are not matching the mean value. The authors should also add a legend to this table, as it is currently unclear what the values represent (i.e. arithmetic mean, geometric mean, median, ranges, interquartile ranges?)

Reply 5:

We analyzed Cmin on days 1 and 15 in cycle 1 using blood samples obtained 10 h after drug administration. We have added this information in the legend of Table 3.

We apologize for the typographical errors in Table 3. We corrected the arithmetic mean ± SD and ranges of Cmin evaluated on day 1 of 600 mg alectinib administration. In addition, we have added a legend to Table 3 for better comprehension of the values.

Comment 6: It would be interesting to explore if the patients who responded to treatment or had long-term benefit had higher exposure than the patients who did not, although I'm aware you don't have that much PK data. Also, it would be interesting to know whether the patients with DLTs have an exceptionally high PK exposure.

Reply 6:

As indicated by the reviewer, we could not assess the association between efficacy and PK of alectinib owing to a small number of samples.

Two out of 3 patients with DLTs discontinued alectinib prior to the collection of samples for PK analysis in cycle 1 on day 15. On day 15, Cmax (1210 ng/mL) and Cmin (1060 ng/mL) of the patients with DLT (grade 3 Creatine phosphokinase increase) were relatively high. Thus, a high plasma concentration of alectinib might affect the toxicity.

Comment 7: Table 2: are these only treatment-related AEs (i.e. possibly/probably/ definitely related according to CTCAE) or also unrelated AEs? Please specify this further. Also, how many patients needed a dose reduction or discontinued treatment due to toxicity? The authors could specify this in the text.

Reply 7:

Thank you for this valuable comment.

As suggested, we have revised Table 2. The bracketed numbers represent causally related values. The treatment of two out of 6 patients who were administered 600 mg BID

alectinib was discontinued due to DLTs (one patient had grade 3 erythema multiforme, another patient had grade 3 rash and grade 3 hepatic function abnormal). In contrast, the treatment of only 1 out of 28 patients who were administered 450 mg BID alectinib was discontinued due to pneumonitis, and a requirement of dose reduction of alectinib was not observed in any no patients due to toxicity.
We have added this information on page 12, lines 274-278.

Comment 8: What are the IC50 values for RET for vandetanib and cabozantinib? And for selpercatinib (LOXO-292) and pralsetinib (BLU-667)? It would be interesting to compare these with the IC50 value of alectinib (i.e. 4.8 nM).

Reply 8:

The IC50 values of each drug determined by in vitro RET kinase inhibitory activity were reported previously as follows: 4 nM of vandetanib, 11 nM of cabozantinib, 0.4 nM of selpercatinib, 0.4 nM of pralsetinib (Subbiah V, et al. J Clin Oncol. 2020;38:1209-1221.) and 4.8 nM of alectinib (Kodama T, et al. Mol Cancer Ther. 2014;13:2910.). Based on this data, selpercatinib and pralsetinib exhibit 10 times higher inhibitory activity against RET compared to other drugs. The potent activity and selectivity may lead to durable efficacy against cancers involving RET rearrangements (Drilon A, et al. N Engl J Med. 2020;383:825-835.) (Subbiah V, et al. Cancer Discov. 2018;8:836-849). This can be one of the reasons for the limited activity of alectinib in this study.

Minor remarks

Comment 1: The authors concluded that the maximum tolerated dose was 450 mg BID in Japanese patients with RET-rearranged NSCLC. Although the study was performed in line with the traditional 3+3 design, the dose level is often expanded to six patients before deciding on the MTD/R2PD, as was also done in the global phase I study of alectinib. Why did the authors decide not to do this in the current study?

Reply 1:

The design of this study (Supplementary Figure 1) was determined by referring to the safety profile of the AF-002JG study (Gadgeel SM, et al. Lancet Oncol 2014;15: 1119-1128). Although Japanese patients were not enrolled, the safety of 600 mg BID alectinib had been already confirmed in the AF-002JG study. Hence, we thought that MTD/R2PD could be determined in the first 3 patients enrolled in each cohort who did not demonstrate any DLTs. Furthermore, the Japanese regulatory agency (PMDA) advised that our design was acceptable during the planning of this study.

Comment 2: Was alectinib administered under fed conditions (according to the label)?
Reply 2:
Alectinib was administered orally twice per day within 30 minutes after a meal (once in the morning and once in the evening). We have added this sentence on page 9, lines 200-201.

Comment 3: Lines 280-283: the sentence "In the global study … alectinib is administered." seems to be out of place here? Please check or rephrase.
Reply 3:
As per the suggestion, we have removed the sentence.

Comment 4: Lines 187-189: was MRI brain performed?
Reply 4:
The brain was assessed by CT-scan or MRI. We have added this information on page 9, line 216.

Comment 5: Lines 179-180: "continued until …" only in part 2 or also in part 1?
Reply 5:
As suggested, we have corrected the sentence as follows (page 9, lines 201-203).
"In phase 1, alectinib (cohort 1: 600 mg, cohort 2: 450 mg) was administered in a 21-day cycle until the criteria for respite, dosage reduction, or discontinuation of the treatment protocol were met."

Comment 6: In the abstract the authors use P1 and P2, while in the manuscript they use step 1 and step 2, it would be more clear to use one term throughout the manuscript.
Reply 6:
As recommended, we have replaced step 1 with phase 1, and step 2 with phase 2 throughout the manuscript.

Comment 7: The sample size calculations were based on an expected ORR of 60%, while previous studies with cabozantinib and vandetanib showed lower ORRs (i.e. 28% and 53%, respectively). What was the rationale to choose an ORR of 60% for the sample size calculations?
Reply 7:
We determined the expected ORR according to the reported efficacy (ORR 60-70%) of

gefitinib in the EGFR mutant lung cancer (Maemondo M, et al. N Engl J Med 2010; 362:2380-8.) and crizotinib in the ALK-rearranged lung cancer (Kwak EL, et al. N Engl J Med 2010; 363:1693-703.). These tyrosine kinase inhibitors were the first drugs approved globally in NSCLC patients with each driver mutation.

Comment 8: First paragraph of the Introduction section: it might be valuable to add the incidences of the subtypes of lung cancer and the specific driver mutations in brackets.

Reply 8:

As suggested, we have revised the sentences as follows on page 5, lines 80-89, and have added the reference (Kohno T et el. Transl Lung Cancer Res. 2015; 4:156-64) as Ref 1.

"Lung cancer is the leading cause of cancer deaths worldwide. Histologically, it is subdivided into small cell lung cancer and non-small cell lung cancer (NSCLC). Non-small cell lung cancer accounts for ~80% of cases of lung cancers, with adenocarcinoma being the most frequent subtype. Several driver oncogenes and mutations, such as epidermal growth factor receptor (EGFR) mutations in 40 to 55% cases, anaplastic lymphoma kinase (ALK) rearrangement in 3 to 5% cases, ROS1 rearrangement in 2 to 3% cases, v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutation in 0.5 to 1% cases, and neurotrophic tropomyosin receptor kinase (NTRK) rearrangement in 1% or fewer cases, have been identified in East Asian lung adenocarcinoma patients (1). Individualized therapy based on gene profiling with corresponding targeted drugs has been introduced into clinical practice (2-8)."

Comment 9: Line 100: "PFS of 5.5 months (1-69)" – what does (1-69) represent, is this a range or confidence interval?

Reply 9:

We apologize for this typographical error. It indicates the number of references. We have corrected the sentence (page 5, line 111).

Comment 10: Lines 100-103: "These results were not comparable to …" – please specify on what aspect? I think you mean that you would expect higher efficacy if you manage to target RET effectively, like we see for EGFR and ALK, but please rephrase this.

Reply 10:

As suggested, we have corrected the sentence as follows on page 5, lines 112-115.

"These results demonstrate that the efficacy of these drugs was lower compared to those in NSCLC cases with EGFR mutations or ALK rearrangement treated with EGFR-tyrosine kinase inhibitors (TKIs) or ALK-TKIs."

Comment 11. Line 117: “assess the maximum efficacy of alectinib” – I would rephrase this to “determine the objective response rate”

Reply 11:

As suggested, we have corrected the sentence on page 6, lines 134-135.

Comment 12. Lines 110-112: “ In a Japanese phase I trial…” – please specify that this was in ALK-positive patients.

Reply 12:

As suggested, we corrected as follows (page 6, lines 123-125).

“In a Japanese phase 1 trial in ALK-rearranged NSCLC patients, alectinib at 300 mg twice a day (BID) showed remarkable efficacy with a response rate of 93.5% and median PFS of longer than 27 months without any dose-limiting toxicity (DLT).”

Comment 13: Line 126: “Primary endpoints were DLT… “ – I would rephrase this to “Primary endpoint was to establish the maximum tolerated dose …”

Reply 13:

As suggested, we have corrected the sentence on page 7, line 142.

Reviewer C

Comment 1: It would be interesting to see a more detailed description about sample size calculation (as well as about the statistical analysis plan) in the body of the manuscript.

Reply 1:

Following the reviewer's comments, we have added a paragraph of "Statistical Analysis" in the Materials and Methods section as follows (page 7, lines 149-160).

“In phase 1, the sample size was determined based on a 3 + 3 phase 1 design for oncology drugs. In phase 2, the planned sample size of 17 RET-TKI-naïve patients was determined to reject a null ORR of 30% at a one‐sided significance level of 0.05 under an expected ORR of 60% with a power of 0.80. In addition, a maximum of 10 patients previously treated with other RET-TKIs were also enrolled for exploratory analysis in phase 2. In phase 1, DLT rate was evaluated using a 3 + 3 design, and the recommended dose was determined. All patients in phase 2 and RET-TKI-naïve patients treated with RD of alectinib in phase 1 were included in phase 2 efficacy analysis. We estimated the confidence interval (CI) of the ORR based on the exact binomial distribution with a one‐sided significance level of 5%. In phase 2, the treatment was deemed promising if the estimated lower limit of the ORR exceeded the threshold value of 30%. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).”

Comment 2: Did all enrolled patients remain in the study without protocol deviations? It would be interesting to report clearly (maybe in a small flow chart) the number of patients screened, enrolled and drop outs (along with the reasons), as well as the adopted methods to handle with missing data (if this is the case).

Reply 2:

Thank you for this valuable comment. All patients remained enrolled in this study without protocol deviations.

Reviewer D

Comment 1: Despite pre-clinical activity of Alectinib in vitro and in vivo, the study showed limited activity and as you wrote in line 297 the reason is unclear. In this regard the question is, if the patient who achieved an objective response and other 13 patients with SD have had any other molecular features differing them from the rest of the group e.g. variant of fusion, co-drivers, additional histological components (e.g. squamous, NEC) etc.?

Reply 1:

Thank you for this valuable comment.

All patients enrolled in this study had adenocarcinoma (page 11, line 237). To make this information clearer, we have added information of histology in Table 1. In addition, all patients were determined negative for EGFR mutation and ALK rearrangement (Page 8, lines 170-171). As shown in Figure 1A, the RET-fusion partner was not associated with the efficacy of alectinib. However, as indicated by the reviewer, co-alterations of other oncogenes and/or variants of RET fusion might affect the efficacy of alectinib. Unfortunately, we did not analyze them in this study.

Comment 2: Can you please clarify about the RET status of those four patients with unknown fusion partner: are you sure that the RET-gen was found in a rearranged condition? Did you confirm it by RNA Archer panel or others?

Reply 2:

In this study, multiplex RT-PCR, NGS, and break-apart FISH were defined as eligible for patients whose tumor was positive for RET-rearrangement, which was confirmed by at least 2 out of 3 different assays. Of these assays, multiplex RT-PCR was designed with multiple primer sets to detect 1 of the 8 RET fusion variants, including 7 variants of KIF5B-RET (K15;R12, meaning KIF5B exon 15−RET exon 12, K16;R12, K22;R12, K23;R12, K24;R8, K24;R11 and K15;R11) and a variant of CCDC6-RET (C1;R12). Thus,

a signal amplified by this multiplex RT-PCR resulted in the detection of one of the 8 variants. However, the variant type was not determined by this assay, since the assay was performed with the multiple primer sets in a reaction mixture. The tumors of the four RET fusion-positive patients whose fusion partners were unknown were determined to be positive by both multiplex RT-PCR and break-apart FISH. However, these tumors were not analyzed by NGS, since the amounts of sample were not sufficient for the NGS analysis. Hence, the fusion partner was not determined in the four patients.

Therefore, we have revised a sentence in Materials and Methods, and added some sentences in Results, as follows.

"RET rearrangements were identified by three different methods, such as multiplex reverse transcriptase PCR (RT-PCR), a break-apart fluorescence in-situ hybridization (FISH) assay, and multiplex genomic diagnostics targeted next-generation sequencing (NGS) systems (Oncomine Cancer Research Panel or Oncomine Comprehensive Assay)." (page 7, lines 163-166).

"Of the 34 patients, 21 had KIF5B-RET and 9 had CCDC6-RET, all of which were determined by NGS. In the remaining 4 patients, RET rearrangements were detected by multiplex RT-PCR, but the variant type was not determined as the samples were not available for NGS. All 34 patients were determined positive for RET rearrangements by break-apart-FISH analysis." (page 11, lines 252-256)

Comment 3: Did these 5 patients previously treated with RET-TKI get performed a rebiopsy for finding resistance mechanism? If they, e.g. developed EMT, no response of Alectinib can be expected.

Reply 3:

As indicated by the reviewer, it is important to explore the resistance mechanisms of RET-TKIs. However, the 5 patients previously treated with RET-TKIs were not subjected to re-biopsy. Therefore, we were not able to analyze their data.

Comment 4: Regarding observed median PFS of 3.4 months and OS of 19 months, can you clarify if any patients have been treated with Alectinib beyond progression and what was their further treatment lines?

Reply 4:

In this study, a continuation of alectinib treatment was not allowed beyond disease progression. Furthermore, we did not collect the information on treatment after the termination of alectinib treatment.

Comment 5: In exclusions criteria the patients with unstable brain metastases were not

allowed to the study. How many of these 34 had stable brain metastases?

Reply 5:

Thank you for this valuable suggestion. Four patients in phase 1 and 4 patients in phase 2 with brain metastases at baseline as determined by the central review board were enrolled in this study. We have added this information in Table 1.

Comment 6: Did you use ctDNA to monitor the response?

Reply 6:

Unfortunately, we did not collect ctDNA in this study.

Comment 7: Line 61 – There are remarkably effective drugs out here like selpercatinib and pralsetinib with solid evidence and some new like RXDX-105 with RR of 67% with non- KIF5B partners. Maybe reconsider in conclusion mentioning about importance of the ongoing research of resistance mechanism for RET-rearranged patients, as we otherwise actually have two very potential drugs for patients with RET-rearrangement.

Reply 7:

Thank you for this valuable suggestion. Considering the reviewer's comment, we have corrected the sentences as follows on page 3, lines 72-74.

"Further investigation to elucidate the mechanisms underlying sensitivity and resistance of RET inhibitors is required to improve the outcomes for these patients."

Comment 8: Line 97 - reconsider to add "modest" or limited" efficacy of Vandetanib or Cabozantinib.

Reply 8:

Thank you for this valuable comment. As recommended, we have added "modest" on page 5, line 109.

Comment 9: Line 112 - can you, please explain " Because of the regulatory reasons", thank you.

Reply 9:

Thank you for this valuable comment. Alecensa formulation contains 50% sodium lauryl sulfate (SLS) as an excipient in comparison with alectinib. The maximum dose of SLS of drugs that had been approved before in Japan was 300 mg a day. Therefore, the sponsor determined that 300 mg BID of Alecensa (=300 mg of SLS) as the maximum dose a day would be appropriate.

We have added these sentences on page 6, lines 125-129.

Comment 10: Line 304 - excellent that the authors mention a novelty in approach of treatment of genomic defined NSCLC with is combing treatment with two TKIs. Beyond HER2, there have been described also other genes like AXL and RAS occurring as resistance mechanism in patients with RET-rearrangement.

Reply 10:

We thank the reviewer for this constructive comment. We have learnt that Nelson-Taylor, S. K et al. reported that the resistance to ponatinib, a multi-kinase inhibitor with RET inhibitory activity, could be emerged by reactivation of RAS/MAPK signaling via NRAS mutation or activation of EGFR and AXL (Mol Cancer Ther 2017;16(8): 1623-1633). We added this information in the Discussion section. We have added this paper as reference 35 and accordingly renumbered the following references. (page 14, lines 337-339).