Peer Review File

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< Respond to the Reviewer A>

Comment 1:

Please add the number of patients who have Ex19 and Ex21 mutations in the abstract separately in between brackets.

Reply 1:

Thank you for your valuable comments. I have added the numbers of patients with Ex19 and Ex21 mutations to the abstract.

Changes in the text:

The numbers of patients with pN1-N2 Ex19 (n=66) and Ex21 (n=63) have been added to the abstract (page 4, line 10-11).

Comment 2:

Please revisit and edit the word "recurrent" in your conclusion. If all group was on recurrent (rather than de-novo) NSCLC cases, this should be better specified in the title and methods.

Reply 2:

Thank you for your valuable comments. In this study, patients with pathological pN1-N2 EGFR-Mt who underwent curative intent surgery were included and analyzed to determine whether RBM10 and PD-L1 expression in IHC affected their prognosis. In addition, we analyzed the effect of RBM-10 and PD-L1 expression on the response to 1st line EGFR-TKI in patients with recurrent pN1-N2 EGFR-Mt after surgery. This point is explained in the Abstract and the Methods section of the main text.

Changes in the text:

In the Abstract of method part (page 4, line 16), following sentence was added: “The effects of RBM10 and PD-L1 expression on progression-free survival (PFS) of EGFR--tyrosine

kinase inhibitors (TKI) therapy among patients with recurrent pN1-N2 EGFR-Mt lung adenocarcinoma (n=67) were examined using log-rank tests."

In the 2.5 Statistical analysis subsection part (page 16, line 11), following sentence was added: "The OS of patients with pN1-N2 EGFR-Mt lung adenocarcinoma and PFS of patients with recurrent pN1-N2 EGFR-Mt lung adenocarcinoma with low/high RBM10 expression and PD-L1 negativity/positivity were analyzed using the Kaplan–Meier method and compared using the log-rank test."

Comment 3:

Section 2.4: Please specify the primary and secondary outcomes here.

Reply 3:

Thank you for your valuable comments. As you mentioned, the primary and secondary outcomes have not been mentioned in the manuscript. We have added the primary and secondary outcomes in Subsection 2.4.

Changes in the text:

In the Method section (page 15, line 9), following sentence was added; The primary outcome of this study was to compare the OS of patients with EGFR-Mt lung adenocarcinoma in RBM10 low/high and PD-L1 negative/positive expression groups. The second outcome was to compare the progression-free survival (PFS) of patients with recurrent EGFR-Mt in the RBM10 low/high and PD-L1 negative/positive expression groups.

Comment 4:

Section 2.5: Please specify "Continuous variables were compared using the Mann–Whitney U test or Student's t-test" eg "based on normality testing. Please add a reference to EZR if possible.

Reply 4:

Thank you for your valuable comments. We have specified the methodology of statistical analysis as you suggested and added the reference for EZR in this manuscript.

Changes in the text:

We have corrected the sentence on page 16, line 9. Moreover, a reference regarding EZR

(26) (Kanda Y. et al Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48:452-8) was added.

Comment 5:

Please specify if there is any missing data and how you managed that,

Reply 5:

Thank you for your valuable comments. All clinicopathological data of the patients in this study were included in our database, and there were no missing data. Some patient prognoses were unknown and were updated through a prognostic survey.

Changes in the text:

None.

Comment 6:

Please report mean follow-up using reversed Kaplan Meier method with those "alive" considered to have the events.

Reply 6:

Thank you for the suggestion. As you suggested, we calculated the follow-up period using the reverse Kaplan-Meier method.

Changes in the text:

An explanation of the reverse Kaplan-Meier method has been added to the Methods section (2.5. Statistical analysis) (page 16, line 14). The median follow-up period (54.7 months) has been corrected in the Results section (page 18, line 16).

Comment 7:

Figure 3: Please add 95% confidence intervals to the curves and try to cut the x-axis at 6 years. You may edit other KM curves (just for consistency but this is up to you).

Reply 7:

Thank you for your valuable comments. We have corrected Figure 3, 4, and 5 as you

suggested: 95% confidence intervals have been added to the KM curves and the x-axes were cut at 6 years.

Changes in the text:

We added 95% confidence intervals to the KM curves and edited KM curves to cut the x-axes at six years in Figure 3, 4, and 5.

Comment 8:

Table 1: Please define low vs high PDL1 and RBM10 in the table footnote.

Reply 8:

Thank you for your valuable comments. We have corrected Table 1 by adding the definitions of low/high RBM10 expression and negative/positive PD-L1 expression to the footnote, as suggested.

Changes in the text:

We have added the definitions of low/high RBM10 expression and negative/positive PD-L1 expression to the footnote of Table 1.

Comment 9:

Please try to investigate progression-free survival, even if variables of interest were statistically insignificant, using Table 2 as a template and add that to supplements.

Reply 9:

Thank you for your valuable comments. Univariable and multivariable analyses were performed using the Cox proportional hazards regression model, and the results are shown in the Supplementary Table.

Changes in the text:

A supplementary Table has been added and the results are included in the Results section of the manuscript (page 20, line 8-10).

Comment 10:

Table 2; Please specify the number of patients in the table title.

Reply 10:

Thank you for your valuable comments. As you suggested, the number of patients has been added to each table title.

Changes in the text:

The number of patients has been added to the titles of Tables 1, 2, and the Supplemental Table.

< Respond to the Reviewer B>

Comment 1:

Please show the rationale why the authors set the threshold for high and low expression of RBM10 at 75%, which seems to be very high. Even in the low intensity samples in fig1 seems to be stained. Are there any samples that are almost negative for RBM10 staining?

Reply 1:

Thank you for your valuable comments. To date, few studies have evaluated the IHC expression of RBM10 in primary lung cancer tissues using the RBM10 antibody No. HPA034972 (Sigma); therefore, there are no criteria for its cut-off value for RBM10 expression. Zhang S et al. classified RBM10 staining intensity into four levels (strong, moderate, faint, and no staining) and defined RBM10-positive as 10% of tumor cells or more being stained at a strong or moderate intensity, while the rest were defined as RBM10-negative; 63 out of 87 lung cancer patients (72.4%) were RBM10-positive (1). In this study, staining intensity was classified into three levels: high, moderate, and low. Low intensity was observed in 42 cases, moderate intensity in 77 cases, and high intensity in 10 cases, with approximately 60% of EGFR-Mt tumor cells showing moderate intensity. Because the mean percentage of tumor cells stained at moderate intensity was approximately 75%, we set the threshold for this study at 75%.

In this study, five samples were almost negative for RBM10 staining (40% or less of the tumor cell cytoplasm was stained at low intensity). Figure 1 shows images of tumor cells that typically stained almost negative for RBM10.

(Reference)

(1) Zhang S, Bao Y, Shen X, Pan Y, Sun Y, Xiao M, Chen K, Wei H, Zuo J, Saffen D, Zong WX, Sun Y, Wang Z, Wang Y. RNA binding motif protein 10 suppresses lung cancer progression by controlling alternative splicing of eukaryotic translation initiation factor 4H. EBioMedicine. 2020 Nov;61:103067.

Changes in the text:

Figure 1 has been revised.

Comment 2:

Even with that threshold setting, the frequency of high and low RBM10 is 2:1, which is considered a very high proportion of high. In the report by Nanjo et al. the expression of RBM10 is shown at the quartile point. Similarly, please show the PFS of OS and EGFR-TKI at the quartile. Also, as the authors indicate, RBM10 mutations are reported to be 8-22%, please show the Os and PFS of EGFR-TKI in the low 25% quartile of RBM10 expression and the highers in 2 curves. If these PFS analyses show no correlation between PFS of EGFR-TKI and RBM10 expression, I recommend weakening or dropping the reference in the Conclusion of the Abstract.
Also, mention that you are using IHC with a very low frequency of low RBM10 expression samples, and please discuss above as much as possible in the discussion part starting from page7 Line19.
You can make part of your response to this comment in a REVIEWER ONLY fig.

Reply 2:

Thank you for the suggestion. As shown in Table 1, high RBM10 expression was observed in 44 (34.1 %) patients. Zhang et al. reported that 63 of 87 lung cancer patients (72.4%) were stained with strong or moderate intensity, with 10% or more tumor cells stained on the IHC section slide. Considering that the tumors in this study included pN1-N2 or advanced lung adenocarcinoma with a high threshold of 75%, we believe that the high RBM10 expression of 34.1% was a reasonable result. A discussion on the frequency of high and low RBM10 expression has been added to the main text (page 22, Line 3–14)

As you suggested, we compared the OS and PFS of EGFR-TKIs between each quartile of RBM10 expression, as analyzed by Nanjo et al.. To rank patients based on RBM10 staining intensity, coefficients were assigned based on the RBM10 staining intensity of the tumor (low=1, moderate=1.5, high=2) and multiplied by the tumor staining percentages. All cases were then divided into the first, second, third, and fourth quartile groups for analysis. As shown in Reviewer Only Figure 1A, the fourth group tended to have a worse OS than the other three groups; however,

the difference was not statistically significant (p=0.064). The fourth group had a significantly worse OS than the first group (p=0.045, Reviewer Only Figure 1B).

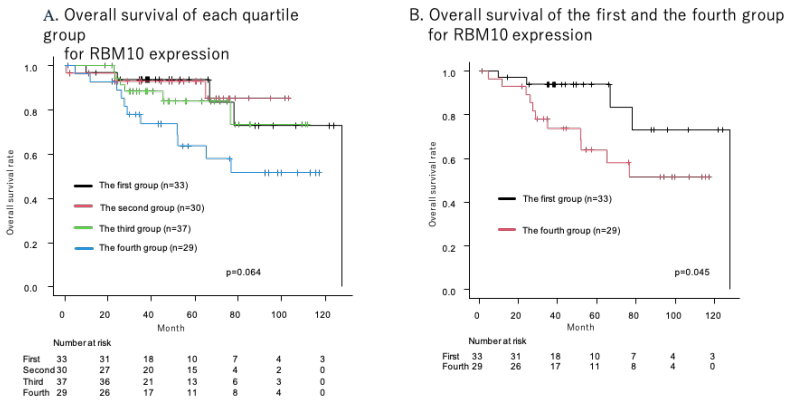
As shown in Reviewer Only Figure 2A, OS was significantly worse in Ex21 lung cancer based on RBM10 staining intensity (p=0.029). The fourth group also had a significantly worse OS than the first group among Ex21 (p=0.016, Figure 2B). As shown in Reviewer Only Figure 3A, no significant difference was observed in the PFS of first-line EGFR-TKIs based on RBM10 staining intensity. However, as shown in Reviewer Only Figure 3B, the fourth group tended to have a worse PFS than the first group.

Taken together, these results suggest that there is an association between RBM10 expression intensity and the OS and PFS of EGFR-TKIs.

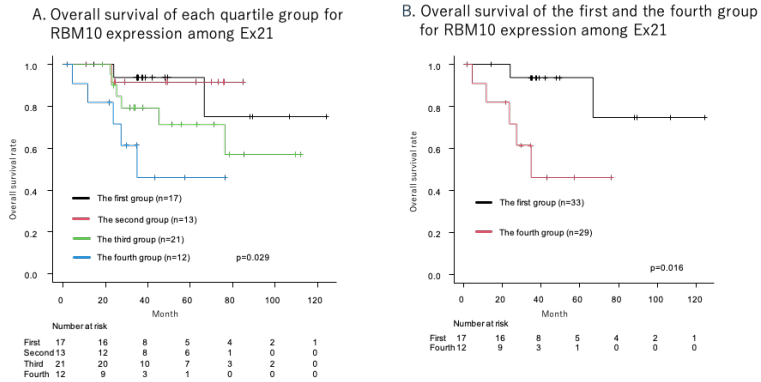
Changes in the text:

We have added a sentence regarding the frequency of RBM10 high expression on page 22, line 3–14 to the Discussion section.

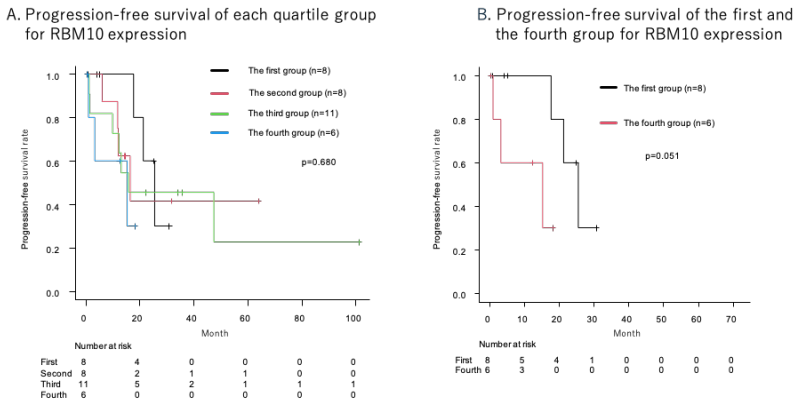
Reviewer only figure 1



Reviewer only figure 2



Reviewer only figure 3



Comment 3:

Nanjo et al. report that RBM10 mutations that result in loss of RBM10 expression are more clustered in EGFR ex21, please present the frequency of RBM10 low in each of EGFR ex19 and ex21 in this study.

Reply 3:

Thank you for your suggestion. Ex21 lung cancer was observed in 44 patients (51.8%) in the low-expression group and in 19 patients (43.2%) in the high-expression group (Table 1). In this study, the number of patients with high or low RBM10 expression did not differ according to the subtype of EGFR mutation (p=0.458).

Changes in the text:

The number and frequency of patients with Ex21 lung adenocarcinoma have been added to page 17, line 18–page 18, line 4, in the Results section of the manuscript.

Comment 4:

Since it is difficult to understand the expression of RNA scope, please explain more in the figure legend, such as the redspot is positive, etc. Also, please indicate the scale bar and magnification on the pictures of fig1 and fig2.If possible, please make the position of RNA scope in fig2 and IHC in fig1 the same. or show the RBM10 IHC of RNA scope samples in Fig2.

Reply 4:

Thank you for your suggestion. We have added an explanation of the RNA scope expression to the legend of Figure 2. Scale bars are shown in Figures 1 and 2, and photographic

magnification is provided in the legends of each figure. We used frozen tissue sections of tumors that have been operated on as recently as possible to evaluate the RNA scope more accurately, thus we were not able to contrast this with the RBM10 IHC in the TMA.

Both RBM10 RNA scope and IHC with TMA evaluated the tumor invasion area, resulting in more mRNA in the cytoplasm of the RBM10 high-expression group and less mRNA in the cytoplasm of the RBM10 low-expression group.

Changes in the text:

Figure 1, Figure 2, Figure 1 legend, and Figure 2 legend have been corrected.

Comment 5 :

For RBM10 mutations and metastasis, see ″Lengel HB, et al. Genomic mapping of metastatic organotropism in lung adenocarcinoma. cancer Cell. 2023;41(5):970-985.e3 ." If you like, consider to cite this where it relates to pN2 metastasis and RBM10.

Reply 5:

Thank you for your valuable comments. I have read “Genomic mapping of metastatic organotropism in lung adenocarcinomas. cancer Cell. 2023;41(5):970-985.e3." by Lengel HB et al.

We have a better understanding of RBM10 mutation; RBM10 mutation are more common in non-metastatic early stage lung adenocarcinoma. As you suggested, we have discussed in the Discussion section of the manuscript where it relates to pN2 metastasis and have included Lengel HB’s manuscript as a reference.

Changes in the text:

We have added this to the Discussion on page 23, line 5-8.

< Respond to the Reviewer C>

Comment 1 :

PD-L1 was evaluated using clone E1L3N. Whilst IHC assays using clones 22C3, SP142, SP263 etc are clinically validated for use in NSCLC, E1L3N is not. Can authors justify reasons for use of this clone and/or correlation of its performance with other validated clones.

Reply 1:

Thank you for your valuable comments. The clone E1L3N used in this study showed a high concordance rate with 22C3(1)(2), SP142(3), SP28-8(3), and SP263(4), which are commonly used in clinical practice. Therefore, we believe that the use of clone E1L3N for PD-L1 evaluation in this experiment-based study was acceptable.

(Reference)

(1) Zhang W, Cao Z, Gao C, Huang Y, Wu C, Zhang L, Hou L. High concordance of programmed death-ligand 1 expression with immunohistochemistry detection between antibody clones 22C3 and E1L3N in non-small cell lung cancer biopsy samples. Transl Cancer Res. 2020 Oct;9(10):5819-5828.

(2) Xu H, Dong X, Zhao H, Hou T, Chen C, Chen G, Ye J, Li Y. Clinical evaluation of a laboratory-developed test using clone E1L3N for the detection of PD-L1 expression status in non-small cell lung cancer. J Clin Lab Anal. 2021 Mar;35(3):e23696.

(3) Sheffield BS, Fulton R, Kalloger SE, Milne K, Geller G, Jones M, Jacquemont C, Zachara S, Zhao E, Pleasance E, Laskin J, Jones SJ, Marra MA, Yip S, Nelson BH, Gown AM, Ho C, Ionescu DN. Investigation of PD-L1 Biomarker Testing Methods for PD-1 Axis Inhibition in Non-squamous Non-small Cell Lung Cancer. J Histochem Cytochem. 2016 Oct;64(10):587-600.

(4) Munari E, Zamboni G, Lunardi G, Marconi M, Brunelli M, Martignoni G, Netto GJ, Quatrini L, Vacca P, Moretta L, Bogina G. PD-L1 expression in non-small cell lung cancer: evaluation of the diagnostic accuracy of a laboratory-developed test using clone E1L3N in comparison with 22C3 and SP263 assays. Hum Pathol. 2019 Aug;90:54-59.

Changes in the text:

None.

Comment 2:

Data on adjuvant chemotherapy was not provided. It is not clear the study period – were any patients also given adjuvant EGFR TKI? This information needs to be provided & included in the analysis as it would impact on outcomes of treatment in this cohort of patients, esp in context of a retrospective series where patients are not treated uniformly

Reply 2:

Thank you for your valuable comments. Postoperative adjuvant TKI was approved in

Japan during the study period of between 2010 and 2020. Patients who received adjuvant EGFR-TKI were not included in this study. In Japan, osimertinib was approved as postoperative adjuvant therapy in September 2022 based on the ADAURA results, so further analysis is needed to determine the impact of RBM10 and PD-L1 on adjuvant osimertinib. We will continue to investigate the impact of RBM10 and PD-L1 on adjuvant osimertinib.

Changes in the text:

In the Methods section (2.1 Patients), we added a statement stating that patients who received adjuvant EGFR-TKIs were excluded from this study (page 12, line 11).

Comment 3 :

Primary and secondary objectives of the study are not clearly stated

Reply 3:

Thank you for your valuable comment. As you mentioned, the primary and secondary objectives of this study were not stated in the main text of the manuscript. We have added the primary and secondary objectives in Subsection 2.4.

Changes in the text:

We have added an explanation of the primary and secondary objectives in Subsection 2.4 (page 15, line 9-13).

Comment 4 :

In the results section, the results are presented without any clear subheadings and it is confusing.

Reply 4:

Thank you for your suggestion. As you suggested, we agree that it would be easier for readers to understand if there were subheadings in the Results section.

Changes in the text:

We have added subheadings in the Results section of the manuscript.

Comment 5:

Overall, the study numbers are small and by further dividing into smaller and smaller subgroups, it is hard to draw any firm conclusion from the results presented here.

Reply 5:

Thank you for your suggestion. As you mentioned, the number of patients in this study was small, and the number of patients with recurrence was even smaller. Since we cannot say that high RBM10 expression or PD-L1 expression can predict the effect of EGFR-TKIs in such a small number of patients, we slightly modified the wording in the conclusion and in the Highlight Box.

Changes in the text:

In the Abstract, we have corrected the last sentence to "In patients with recurrent pN1-N2 EGFR-MT lung adenocarcinoma, PD-L1 and RBM10 expression may influence response to EGFR-TKIs.". The sentence in Highlight Box was also corrected. In addition, the text in the limitation section was revised (page 27, line 5).

Comment 6:

In addition, no data is supplied on the type of EGFR TKI used (1st vs 2nd vs 3rd gen) in the context of recurrence, which could also impact outcomes.

Reply 6:

Thank you for your valuable comment. As you mentioned, information on the generation of EGFR-TKIs used in first-line treatment is not shown. We have added information on the generation of EGFR-TKIs to Table 1. As shown in the revised Table 1, the number of third-line EGFR-TKIs was not significantly different between the RBM10 high/low expression group and the PD-L1 positive/negative expression group.

Changes in the text:

We have added information on generation of the EGFR-TKI in Table 1.

Comment 7:

Study is overall not practice changing – numbers are small, with wide confidence intervals. Some of the information is not novel (eg, PD-L1 association in EGFR mutant resected

NSCLC has been reported as mentioned by authors)

Response 7:

Thank you for your valuable comment. I agree with Reviewer C that our study was based on a small number of patients and it is difficult to change clinical practice from the findings of the present study. We have added sentences about this in the limitation and revised sentence in Highlight Box. However, we believe that this study will shed light on RBM10 and PD-L1 in predicting the prognosis of patients after curative surgery for pN1-N2 EGFR-Mt lung cancer, as well as the efficacy of EGFR-TKI. Recent ADURA results have also led to the use of EGFR-TKIs as adjuvant therapy after surgery for EGFR-Mt lung cancer. We believe that it will be very important to elucidate which patients respond well to EGFR-TKIs, and that RBM10 and PD-L1 are predictors of which patients will benefit more from EGFR-TKIs. We are planning large-scale prospective clinical studies to determine the impact of RBM10 and PD-L1 on the prognosis and EGFR-TKI efficacy in patients with recurrent disease and in those eligible for postoperative adjuvant therapy.

Changes in the text:

The sentence in Highlight Box was corrected.

We have highlighted in yellow the parts where content has been added or corrected in the manuscript. If you have any other questions, we would like to answer you at any time. Thank you.