Peer Review File

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Reviewer A

Overall, the study design is okay and the manuscript is pretty written in good format. However, there are few major and minor concerns (as below) need to be fixed in the current contents before the manuscript could further consider for publication.

- The authors need to provide the IC50 of osimertinib and abemaciclib for each cell line.
  Reply: We thank the reviewer for the comment. In response to it, we have included the IC50 values for each cell line in Figure 4B and Supplementary Figure 4C.
- How about the MET amplification cell type? I think this is an important one.
  Reply: We appreciate the insightful comment from the reviewer. In response, we have evaluated the efficacy of the combination of osimertinib and abemaciclib against H820, an *EGFR*-mutant cell line with MET amplification. Consistent with data from other *EGFR*-mutant cell lines, the combination of osimeritinib and abemaciclib inhibited the cells effectively, whereas little effect was observed when either agent was used alone on the resistant cell lines. We have included this data in Supplementary Figure 3 and provided a description on page 13, line 281 - page 14, line 288.
- In addition, the TKIs resistance cell lines should also be included in this study to make the whole research findings be more completed.
  Reply: We thank the reviewer for the suggestion. In response to the comment, we have established an osimertinib-resistant cell line derived from EGFR-mutant PC-9 cells through long-term exposure to osimertinib. The resistant line, designated as PC-9/OsiR, is able to survive in the presence of 500 nM osimertinib. Different from data from other EGFR-mutant cell lines including H820, The combination was not effective against PC-9/OsiR cells, which were the acquired resistance model for osimertinib We have included this data in Supplementary Figure 3 and provided a description page 13, line 281 - page 14, line 288
- What is the actual mechanism of the proposed combination treatment? It looks like some key mechanistic experiments are currently missing.
  Reply: We thank the reviewer for the comment. We found that the cell cycle in *EGFR*-mutant NSCLC cells was not completely halted under exposure to EGFR TKI treatment (Figure 1A). CDK4/6 inhibitors, by inhibiting the remaining cell cycle activity, enhanced the effects of osimertinib, which is considered to be the mechanism underlying the combination treatment. The raining cell cycling was maintained by ERK pathway. However, as the reviewer pointed out, the detailed mechanism of the remaining cell cycle under EGFR TKI treatment remains unclear, and this is one of the limitations of our study. We discuss this limitation in the Discussion section (page 16, line 350 - 353).
- Suppl Figure 1, why use DMSO as control? What is the % did the authors use? Please

clarify.

Reply: All the drugs used in the in vitro experiments of this study were dissolved in DMSO. We used DMSO as a control in order to rule out the potential effects of DMSO itself. The concentration of DMSO for each drug or control was 0.1%. We have described this information in the legend of Supplementary Figure 4 (previous Supplementary Figure 1) (page 26, line 585).

- A graphical abstract for this study is required.

Reply: We appreciate the comment. In response to it, we have added the graphical abstract for this study as Figure 7 and described it in the Discussion section (page16, line 331-334).

- I believe some recent published papers could be used as references for the authors to further polish the contents. For examples:

DOI: 10.1007/s00018-022-04647-x https://doi.org/10.1016/j.semcancer.2022.12.006

Reply: We thank the reviewer for the comment. We have cited the referenced paper in our manuscript (page6, line 101, ref. number 9)

Reviewer B

The manuscript by Hara et al describes the significance of targeting CDK4/6 together with the EGFR TKI Osimertinib to effectively kill EGFR mutant NSCLC cells. The authors conducted studies using five different, in vitro cultured, EGFR mutant NSCLC cells and in vivo mouse xenograft models to determine if CDK4/6 inhibitors enhance the efficacy of Osimertinib. Their studies on the NSCLC cells showed that treatment with 100nM Osimertinib does not completely eliminate the cells but when combined with abemaciclib, a CDK4/6 inhibitor, the efficacy of Osimertinib is enhanced. According to the authors, CDK4/6 mediated phosphorylation of Rb and its inactivation happens prior to the development of TKI resistance, and inclusion of CDK4/6 inhibitors enhance the Osimertinib efficacy prior to the acquired resistance development. Furthermore, based on their results from the MEK inhibitor treated NSCLC cells they conclude that ERK activation is responsible for the residual Rb phosphorylation observed in the Osimertinib treated cells. These are all correlative studies and additional studies are required to make such a conclusion. For example, if they knockdown ERK in the cells do they see inhibition of Rb phosphorylation? Does the knockdown of CDK4/6 or ERK make these cells more sensitive to Osimertinib treatment? Is ERK directly phosphorylating Rb? Can ERK affect CDK4/6 activity? What is the status of Rb in Osimertinib resistant cells? Are CDK4/6 inhibitors effective against the resistant cells? Overall, the in vivo results support their conclusion, but the in vitro results need additional clarifications. A few specific comments are provided below for the authors:

Major:

1. How is the 100nM Osimertinib concentration selected for the various cells, is this based on an IC50 analysis because various EGFR mutant NSCLC cells respond to Osimertinib differently? Inclusion of IC50 values/curves for Osimertinib in the cell

lines mentioned in the manuscript would be helpful.

Reply: The reviewer is right. We selected a concentration of 100 nM for osimertinib in order to sufficiently inhibit all the cell types investigated, based on IC50 analysis. In response to the comment, we have included the IC50 values for each cell line in Figure 4B and Supplementary Figure 4C.

2. To conclude that ERK inhibition brings down the residual Rb phosphorylation observed in Osimertinib treated cells, the authors need to conduct a knockdown study using siRNA to ERK. Also, is the sensitivity of the NSCLC cells to Osimertinib enhanced upon knockdown of CDK4/6 or ERK? These studies will strengthen their findings and improve the manuscript tremendously.

Reply: We appreciate the important suggestions from the reviewer. In response to the comment, we have knocked down ERK1 using two individual siRNAs and found the residual Rb phosphorylation was further inhibited by ERK1 knockdown (Supplementary Figure 2B). Furthermore, siCDK4 or siERK1 enhanced the efficacy of osimertinib (Supplementary Figure 2A and 2B). We describe those data in the Result section (page11, line 227-230 and page12, line 253-257).

3. Figure 2B, cell cycle analysis, shows an additive effect only in PC9 and H1975; it appears that the HCC827 and 11-18 combination treatment results are very similar to that seen with Osimertinib alone, please confirm. This is true for the data provided in Supplementary Figure 1B as well, where only PC9 shows an additive effect. HCC827 combination result is very close to that seen with Osimertinib alone, and 11-18 and H1975 results are like Palbociclib single agent results; please clarify these and provide an explanation.

Reply: We thank the reviewer for the comment. As the reviewer pointed out, the results of the combination treatment with HCC827 and 11-18 were very similar to those observed with either drug alone. However, we did see a numerical increase in the percentage of cells in the G0/G1 phase in the cells treated with the combination, which was confirmed through three separate experiments. We acknowledge that the difference was subtle. To avoid overstating the result, we have revised the description to read, ' Although the overall effect was consistent with data from PC-9 and H1975 cells, the increase in cell cycle arrest at the G0/G1 phase in both HCC827 and 11-18 cells was subtle when osimertinib was combined with abemaciclib. ' (page12, line 233-237).

4. The authors talk about CDK4/6 inhibition having a positive impact on the NSCLC cells prior to Osimertinib resistance development, does this mean that the cells do not develop resistance if CDK4/6 is inhibited or delay the development of resistance? What happens if they inhibit or knockdown CDK4/6 in Osimertinib resistant EGFR mutant NSCLC cells, do they become sensitive to Osimertinib?

Reply: We appreciate the insightful comments from the reviewer. Examining Figure 4A, the combination of osimertinib and abemaciclib demonstrated more profound cell growth inhibition compared to either drug alone. However, some residual cells were still observed in the combination treatment, suggesting that the efficacy of the combination is not curative. Based on these results, we believe that completely preventing acquired resistance through combination therapy could be challenging.

As for the osimertinib-resistant cells, we inhibited CDK4/6 with abemaciclib in two *EGFR*-mutant, osimertinib-resistant cell lines (H820; EGFR ex19 and MET amp. and PC-9/OsiR; Acquired resistant cells derived from PC-9 through chronic exposure to osimertinib). The combination of osimertinib and abemaciclib inhibited H820 cells effectively, whereas little effect was observed when either agent was used alone on the resistant cell lines. The combination was not effective against PC-9/OsiR cells, which were the acquired resistance model for osimertinib (Supplementary Figure 3A and 3B). We have described this data on page13, line 281 - page14, line288.

5. Figure 4C; the authors mention that this shows combination index and fraction affected but it appears to be the same panel as shown in Figure 4B top panel, please clarify.

Reply: We deeply apologize for the oversight. We had mistakenly presented the wrong figure in Figure 4C, and have now replaced it with the correct one.

Minor:

1. The manuscript has several grammatical errors or statement errors and need to be edited thoroughly.

   Reply: We are terribly sorry for that. In response to the comment, the manuscript has been reviewed by native English speakers.

2. Figure 3, legend title needs to be modified to read 'RB phosphorylation' instead of 'RB activities'.

Reply: We have modified that in response to the comment (page 23, line 508).

3. Figure 6B, if the authors take a pRb to total Rb ratio it seems to be higher in H1975 and H3255 compared to HCC827; how does this impact the cell cycle and doubling time seen here in Figure 6A, especially with H3255?

Reply: We agree with the comment. A pRb to total Rb ratio seems to be higher in H1975 and H3255 compared to HCC827 and higher ratio of it may be associated with longer doubling time. However, the ratio of 11-18, whose doubling time is relatively short, also seems high. We consider pRB or total RB itself would be better associated with the doubling time compared to the ratio.

4. Line 355, the statement '….. mechanisms, such as C797S, MET and BRAF…., it appears that the authors are mentioning the C797S mutation in EGFR, if so, please mention that.

Reply: We thank the reviewer for noticing that. According to the cited study, CDK4/6 inhibitors overcame acquired resistance caused by C797X secondary mutation, MET amplification, or BRAF secondary mutation. We modified the sentence in response to the comment (page 16, line 355 – page 17, line 356).

Reviewer C

In the manuscript by Hara N, et al., CDK4/6 signaling attenuates the effect of epidermal

growth factor receptor tyrosine kinase inhibitors in EGFR mutant non-small cell lung cancer. The authors provided that the CDK4/6-RB signal axis has an important role in the efficacy of EGFR-TKIs. The manuscript is interesting, well-written, and has a good composition of figures.

Minor concern:

1. In the figure1, the authors should provide the histogram of FACscan as figure 2
   Reply: Figure 1 presents the average values obtained from three FACS analyses, and the differences were statistically analyzed. As a result, the data could not be displayed as a histogram in this figure. However, we understand that a histogram should be provided. In response to the comment, we have included a representative histogram in Supplementary Figure 1.
2. The legibility of the figure is low because the description of the figure is small.
   Reply: In response to the comment, we have increased the size of the annotations in the figures to enhance readability.

Reviewer D

Hara N. et al. showed that the CDK4/6-RB signal axis attenuates the efficacy of EGFR-TKIs in EGFR mutant NSCLC and combined treatment with CDK4/6 inhibitor and EGFR-TKI could be a novel treatment strategy for TKI-naïve EGFR mutant NSCLC.
It is a quite interesting study, but there already have been several studies suggesting the relationship between CDK4/6-RB signaling and EGFR-TKI response. (see Nature Genetics volume 49, pages1693–1704 (2017) and Scientific Reports, 09 Feb 2022, 12(1):2167, etc) Further, La Monica S. et al., demonstrated that the osimertinib-resistant PC-9 and HCC827 cell lines maintained Rb phosphorylation in the presence of osimertinib and were sensitive to the cytostatic activity of the CDK4/6 inhibitor abemaciclib. They also showed that the combination of abemaciclib with osimertinib significantly inhibited the onset of resistance in long-term experiments in osimertinib-sensitive PC9, PC9T790M, and H1975 cells.
In general context, this study is very similar to the work of La Monica S. et al. although it has some new findings. Considering that even the clinical trial of combined treatment with osimertinib and abemaciclib is on-going, this study seems to be short of being accepted for publication in TLCR.

Reply: "We appreciate the insightful comments from the reviewer. As the reviewer noted, several studies have reported on the relationship between CDK4/6-RB signaling and EGFR-TKI response. The studies mentioned by the reviewer (Nature Genetics 2017 and Scientific Reports 2022) revealed that the co-occurrence of CDK4/6 alterations is related to de novo EGFR TKI resistance. However, much of the previous research, including the studies mentioned above, has primarily focused on CDK4/6 and EGFR inhibitors in the context of EGFR-TKI resistant models, without addressing susceptibility models. We have emphasized this point in the document to convey this point more clearly (page 6, line 111-112).

As the reviewer highlighted, the study by La Monica S. et al. did touch upon the susceptibility model. However, the primary focus of their research was the acquired resistance model, and they only explored the cell growth inhibition of abemaciclib and osimertinib in an EGFR-TKI sensitive model.

Unlike the studies mentioned above, our research primarily focused on the susceptibility model to EGFR-TKI, using several EGFR-TKI sensitive cell lines. We detailed the impact of the combination using flow cytometry, genomic CDK inhibition, xenograft models, and more.

We believe our findings are an important complement to clinical studies.