Author Response 1

Reviewer #1

Major comments

1. Comment: ''The authors established the gefitinib resistant cell lines by exposing the cells over time to increasing doses of drug and confirmed resistance using cytotoxicity assays. However, we do not know specifically what resistance pathways these cells have undertaken. For instance, 50% of cells acquire resistance to an exon 20 EGFR T790M mutation, 20% develop MET mutations and or amplifications. MET protein analysis though not changed in this study has been shown in other studies to have high false negative rate. My suggestion is that the authors test for these mutations in all the resistant cell lines using next generation DNA sequencing or RNA sequencing. This is important, how do we know the H1650GR line does not have a T790M mutation which would make using osimertinib the appropriate comparator in the xenograft study?''

Response: Thank you for your comments. The establishment of H1650GR cell line follows the method used in the reference, Han et al., 2015, cited in our paper where the EGFR T790M mutation in the established H1650GR was not reported. (Please see page 4, paragraph 3 of the revised manuscript.)

2. Comment: ''The xenograft result is the key result in this study. The authors do not have an arm with 1650 + gefitinib + miR-30a-5p. I understand that the main aim was to show that dual pathway blockade restores sensitivity to gefitinib but it would be interesting to have seen if upfront blockade has a substantial impact in the gefitinib sensitive setting.''

Response: Thank you for your comments. The purpose of this study is to explore if the combination therapy of gefitinib and miR-30a-5p could overcome the acquired resistance to EGFR-TKIs. Our xenograft result has achieved this aim. In our next study, we will focus on investigating if upfront blockade has a substantial impact in the gefitinib sensitive setting.

3. Comment: ''Figure 7 shows IHC from 1 representative mouse. It would be more useful if the authors could also present a graph with the average values from say 3 mice per group to get an average result on pAKT and PIK3R2 levels.''

Response: Thank you for your comments. The bar chart presents results as the mean over 4 mice in each group has been added (Please see page 18, Figure 7 of the revised paper.)

4. Comment: ''There are a few important papers that the authors have not included in this study. Donev at el (CCR 2011) described that PI3K inihibtion restores gefitinib resistance in xenografts. This is one example.''

Response: Thank you for your comments. We have added some more important references to our manuscript including Donev et al. (2011). (Please see page 19, paragraph 3, line 3 to 5 of the revised manuscript.)

5. Comment: ''There is really no context to this study. What does this study mean in the current context of treatment landscape. I would recommend the authors attempt to discuss this in 1 paragraph''

Response: Thank you for your comments. We have discussed the current treatment of NSCLC, including the traditional platinum-based chemotherapy, the targeted therapies and the third-

generation EGFR-TKI (NCCN Guidelines Insights: Non–Small Cell Lung Cancer, Version 1.2020). (Please see page 2, the last paragraph, and page 3, the first paragraph of the revised manuscript.)

6. Comment: ''Also 4 mice per group is very few. I feel this is very borderline for an adequate statistical analysis. Can the authors please explain why this number was chosen? ''

Response: Thank you for your comments. Based on the power analysis, four mice per group are enough to achieve 80% statistical power for examining the difference. Considering the budget constraint, we finally chose to use 4 mice per group. Age and sex of all the mice are the same. (Please see the first paragraph under "statistical analysis" at page 8.)

Minor comments

1. Comment: ''The first introduction paragraph needs to be revised. This is a very old fashioned introduction. We now have chemo-immunotherapy as standard of care in NSCLC without an actionable driver mutation and the standard of care in EGFRm NSCLC is TKI and in fact now osimertinib. All of this needs to be included. Reference 7 (Shaw et al) is an ALK reference, completely wrong in that sentence.''

Response: Thank you for your comments. We have modified the manuscript according to the comment. The referencing is corrected now. (Please see page 3, paragraph 1, line 10 to 11 of the revised manuscript.)

2. Comment: ''The 2nd paragraph discussing resistance after 1st gen TKIs is also quite weak. MET amplifications, small cell transformation, Pi3K mutations all need to be included here.''

Response: Thank you for your comments. We have modified the manuscript according to the comment. (Please see page 3, paragraph 1, line 7 to 9 of the revised manuscript.)

3. Comment: ''Figure 6A is really hard to read. I would suggest the authors use colours for each treatment group.''

Response: Thank you for your comments. We have specified each treatment by color to enhance the readability. (Please see page 17, Figure 6A of the revised manuscript.)

4. Comment: ''Figure S2 – what are the IC50 values? In microM or nanoM. Wasn't made clear.''

Response: Thank you for your comments. The unit of IC50 is microM, and we have modified the figure according to the comment. (Please see Figure S2 at page 2 of the revised supplementary material.)

Reviewer #2

1. Comment: ''Another instance of incorrect wording of interpretation of a result is listed below: The authors say that "In all this four concentrations of gefitinib, there was expression detected for p-IGF1R, but not for p-MET (Fig. 1B), indicating that the status of IGF1R pathway was activated, and MET pathway was inactivated". The MET pathway was not activated in this case. The interpretation "MET pathway was inactivated" would mislead the readers into believing that MET pathway was active without EGFR inhibitors and inactivated upon Gefitinib treatment.''

Response: Thank you for your comments. The wordings have been corrected accordingly. (Please see paragraph 4 at page 9 of the revised manuscript.)

2. Comment: '' This article should be edited to correct the grammar and incorrectly worded interpretations and results.''

Response: Thank you for your comments.The grammar and wordings of the whole manuscript have been corrected by language editing.

3. Comment: '' The concentration of gefitinib and AEW541 used in Fig. 2 should be indicated.''

Response: Thank you for your comments. The concentration of gefitinib and AEW541 has been added to the caption of Figure 2. (Please see Figure 2 at page 12 of the revised manuscript.)

4. Comment: ''For fig. 3, the specific mutations introduced at the putative miR-30a-5p binding sites within PIK3R2 and PIK3CD must be indicated.''

Response: Thank you for your comments. The mutations are indicated in Figure 3A. (Please see Figure 3A at page 13 of the revised manuscript.)

5. Comment: ''Also, is the expression of miR-30a-5p lower in H1650GR line compared to the parental line, H1650?''

Response: Thank you for your comments. The purpose of this study is to explore if the combination therapy of gefitinib and miR-30a-5p could overcome the acquired resistance to EGFR-TKIs in 1650GR line and xenograft model. The miR-30a-5p originating from the cells is not of our interest in this study. The comparison of the expression levels of miR-30a-5p between H1650GR and H1650 cell lines will be explored in our future study.

6. Comment: ''In addition to luciferase assays, the authors must also perform western blot analysis to show that miR-30a-5p downregulates the expression of PIK3R2 and PIK3CD in H1650R. ''

Response: Thank you for your comments. We have performed the western blot analysis and included the results in Figure 3, at which we found that miR-30a-5p downregulates the expression of PIK3R2 and PIK3CD in H1650R. (Please see Figure 3C at page 9 of the revised manuscript.)

7. Comment: ''Also, can H1650GR be sensitized to Gefitinib by co-treatment with an AKT inhibitor? Also, does the co-blockade of IGF1R in H1650R lines (Fig. 2) that causes reduction in pAKT levels also result in increased killing of this cell line? This would indicate that this cell line is dependent on PI3K/AKT signalling for survival.''

Response: Thank you for your comments. The aim of this study is to explore if the combination therapy of gefitinib and miR-30a-5p could overcome the acquired resistance to EGFR-TKIs. The co-treatment with an AKT inhibitor, and the dependency of cell line on PI3K/AKT signaling for survival will be explored in our future study.

8. Comment: ''Also, the effect of the overexpression of miR-30a-5p alone on H1650 Xenografts must be shown to demonstrate if the combination has superior effect to miR-30-5p overexpression alone. Also, pEGFR/pERK staining should be included in Fig. 7 to demonstrate that Gefitinib effectively inhibited EGFR in vivo in their study.''

Response: Thank you for your comments. The aim of this study is to explore if the combination therapy of gefitinib and miR-30a-5p could overcome the acquired resistance to EGFR-TKIs. Figure 6 results have demonstrated the aim. The effect of the overexpression of miR-30a-5p will be explored

in our future study. We explored the PI3K/AKT signaling pathway, the shared downstream pathway of EGFR and IGF1R, to elucidate their effect on overcoming the acquired resistance.

Since PI3K is the target of miR-30a-5p, and AKT is the downstream of PI3K, they both were stained as shown in Figure 7.