Title: Increased IR-A/IR-B Ratio in Non-small Cell Lung Cancers Associates with Lower Epithelial-Mesenchymal Transition Signature and Longer Survival in Squamous Cell Lung Carcinoma

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Author’s response to reviews: see over
Dear Professor Ian Cree:

Thank you very much for the opportunity to submit a revised version of "Increased IR-A/IR-B Ratio in Non-small Cell Lung Cancers Associates with Lower Epithelial-Mesenchymal Transition Signature and Longer Survival in Squamous Cell Lung Carcinoma (166993693103297). We thank the reviewers for their thorough and critical reviews of the manuscript and for their insightful comments and suggestions, which we have addressed to the fullest extent possible. Following the specific comments from each reviewer, we have stated the rationale for any alterations made to the revised manuscript. Additionally, the changes have been tracked to simplify the review.

We sincerely appreciate your reconsideration of this manuscript and believe that you will find it suitable for publication.

Reviewer 1:

Minor Revisions

1. Abstract, line 9, sentence starting with “Notably…” needs to be modified with correct grammar.

> This sentence has been clarified.

2. The authors should keep the color-coding consistent in the panels of Figure 2 (ie use red for all LUAD samples)

> The color-coding had been modified to be consistent in the panels of Figure 2.

3. In the results the ECM gene signature is referred to as Figure 4C and the EMT gene signature as Figure 4B however in Figure 4, panel B refers to ECM and panel C refers to EMT

> The text has been modified to be consistent with Figure 4.

Major Revisions

1. I think the authors should explain why IGF-IR expression was not evaluated in
This study as there may be some interesting correlations between IGF-IR expression and IR expression. If IGF-IR expression has already been evaluated in these samples, this information should be provided and discussed.

> We did test the mRNA expression levels of IGF1R and found that 12 out of 144 (8%) NSCLC samples have >2 fold IGF1R mRNA expression than the normal lung. We also explored the TCGA RNAseq data. We did not identify any clear relationship between IGF1R and INSR or its isoforms in either of the data sets. Therefore we added this information to the results section, but did not discuss it at length since there were no relationships identified.

2. Since the article is trying to relate the expression of IR-A, IR-B and the IR-A/IR-B ratio to the ineffectiveness of anti-IGFIR therapy in lung cancer, it would have been more useful to measure the levels of IR-A and IR-B in patients that either responded or did not respond to anti-IGFIR therapy. The levels in IR-A and IR-B in lung cancer and their association with prognosis is very interesting but there is no data linking the susceptibility of individuals with different IR expression to their response to anti-IGFIR therapy. If data linking IR and IGF-IR is not provided, I think the manuscript needs to be re-written to simply focus on the importance IR expression (ie remove paragraph in introduction and discussion talking about resistance to anti-IGFIR agents). I kept looking for data linking IR expression to IGF-IR therapy resistance.

> Since we do not have clinical data from patients treated with anti-IGF therapy, we have re-written the manuscript to focus on the importance of IR expression in this study, as suggested by this reviewer. We revised the introduction and discussion accordingly.

3. On the bottom of page 14 and top of page 15, the author is trying to correlate the HIR observed in luminal B breast cancer with EMT. I don’t think this is an accurate comparison. The main difference in luminal A and luminal B breast cancers is the higher level of markers associated with proliferation in luminal B breast tumors. Basal-like and claudin-low breast cancers express markers of EMT however luminal A and luminal B tumors primarily express luminal markers. Therefore, the poorer survival of patients with luminal B tumors compared to those with luminal A tumors in not typically associated with EMT.

> These paragraphs had been modified and the comparison of HIR in breast cancer with EMT has been removed.

Reviewer 2:

Major point

The discrepancy between the results obtained from the RNA-seq database and those obtained from the two independent panels regarding IR-A expression raises important questions. It has been well
characterized that IR-A and IR-B are produced from the alternate splicing of a unique IR pre-mRNA and that the balance between the two isoforms may be altered during pathogenesis including cancer. Up to now, increased IR-A/IR-B ratio has been reported in cancer cells and tissues as a result of IR-A overexpression associated to IR-B downregulation, thus reflecting alterations in IR splicing.

> The reviewer raised a good question about the differences observed between the RNA-seq data and those from qRT-PCR. We had the same question and therefore used QC/QA to exclude the likely possibility that the bimodal distribution of the IR-B expression from the RNA-seq is due to artifacts in RNA-seq data processing. We extensively analyzed the RNA-seq data from the other cancer types and observed similar pattern (see Figure 6). However, as we do not have the tissue samples which were sequenced by RNA-seq, we cannot evaluate further.

Nevertheless, we observed highly significant changes in IR-B gene expression in both RNA-seq and qRT-PCR data, compared to those changes in IR-A (also see the attached figures below).

Noticeably, many of the previously published IR-A/IR-B studies (Frasca F et al. 1999, Chettouh et al. 2013) used the IR-A proportion as the metrics, as illustrated in the figure below. As \( \%\text{IR-A} = 1 - \%\text{IR-B} \), \( \%\text{IR-A} \) is negatively associated with \( \%\text{IR-B} \).
Other points

1. What is the status of IR-A in tumor samples from other tissues?

> As mentioned before, we would like to highlight the significant drop in the IR-B expression in some cancer patients, whereas the gene expression of IR-A in most cancer tissues is comparable to the normal control.

2. In the discussion, the authors argue that it is not clear how the differences in expression levels of IR-A and IR-B are regulated in cancer. To address this point, they evaluated promoter
methylation and found no variation. This is not surprising since alterations in the regulation of IR alternative splicing have been described in other tumors such as liver tumors (Chettouh, Cancer Res, 2013).

> The authors thank this reviewer for pointing this out; we modified the discussion accordingly.

3. How higher IR-A/IR-B ratio may push cells to depend more on IGF-II signaling through IR-A since IR-A tends to be down-regulated in tumor panels (discussion section)?

We added a sentence in the discussion to explain that this is caused by the high affinity of IR-A to IGF2.

4. Reference 15 is incorrect in the discussion.

Reference 15 has been corrected.

5. Too much abbreviations are used in Figure 6 and Table 1.

We had revised Table, and we added the full name of diseases in the table 1, this will help with the abbreviations in Figure 6.

We believe that we have successfully addressed all comments from the reviewers. If you have any questions or need additional clarification, please contact us at your earliest convenience.

Regards,

Jiaqi Huang, PhD