Author’s response to reviews

Title: The Role of Cytochrome c Oxidase Subunit Va in Non-small Cell Lung Carcinoma Cells: Association with Migration, Invasion and Prediction of Distant Metastasis

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Author’s response to reviews: see over
To the honorable editor,

I am returning you the revised form of the previously submitted manuscript for your review and approval. We have studied the reviewers’ comments and have revised the manuscript accordingly.

We rewrote the background section, especially the second paragraph, and corrected the references that were considered inadequate. We also avoided emphasizing the importance of membrane-bound proteins in the background section of the manuscript, although our study target was one of them.

The small defects in the method and result section were corrected. However, we did not change the data of migration and invasion study according to one reviewer’s suggestion, which proposes changing the medium in the insert well, because such a condition was following the manufacturer’s guide and was actually the same in all experiments. Thus the differences in migration and invasion should be considered deriving from the cells themselves rather than the effect of medium.

Regarding the knockdown experiment, we clearly indicated the control, scramble and knockdown cells in the revised manuscript to facilitate the readers to distinguish the difference. Because we used the CL1-5 cells as the representative, the data of H2009 was therefore not present in Fig.5 and Fig.6.

We also evaluated the expression condition of Bcl-2 and added the results in the
manuscript and Fig.7. We hope this can help to improve the mechanistic insight of our current study. Meanwhile, all data of RT-PCR were supplemented with quantification plots to facilitate the readers to interpret the data.

To fit the changes of whole manuscript, we also made some small changes in the abstract part. However, it will not affect the conclusion of the study at all.

Finally, the authors deeply appreciate the editor for the opportunity to response the reviewers’ comments and to improve this article.

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To Reviewer 1:

Major Compulsory Revisions

1. “Background” contains several problems:
   - The authors cited ‘Mol Cell Proteomics 2010;9:1100-17.’ to explain usefulness of 2-D analysis, but I do not think this cite is proper choice.
     → We thank the reviewer’s opinion and have replaced it.
   - I do not understand the importance of membrane proteins which was mentioned out of nowhere in the same paragraph.
     → We agreed with the reviewer’s viewpoint. Although COX Va is a membrane-bound protein, it should not be over-emphasized in the background. We have rewritten the second paragraph of background section.
   - Sentences in 2nd paragraph of ‘Background” must be reorganized because it is lack of logical connection.
     → Thanks for the reviewer’s comment. We have rewritten the second paragraph of background section.
   - In page 6, 17th line: Full names of ‘two more NSCLC’ cell lines should be identified.
     → We have provided the full name of the cell lines.

2. “Methods”
   - In page 7, 17th line: There are no ‘H2126’ or ‘H2009’ cell lines in ATCC. Full names of the cell lines must be recorded.
     → The H2126 is actually ATCC No. CCL-256, and the H2009 is ATCC No. CRL-5911. We have provided the full name of the cell lines in the manuscript, and thanks again for the reviewer’s carefulness.
   - Ref.17 does not contain ‘Migration assay’.
     → It was our neglect. We thank the reviewer’s opinion and have corrected this mistake.
   - In ‘Migration assay’ and ‘Invasion assay’, the authors added 10% FBS containing RPMI media to insert well, but I do not understand why the authors used 10% FBS containing RPMI media because this media could promote proliferation as well as
migration or invasion. I recommend the authors will use 0.5% BSA containing media in insert well.

→ The condition was following the manufacturer’s guide and was actually the same in all experiments. The differences in migration and invasion should therefore be considered deriving from the cells themselves rather than the effect of medium.

3. “Results”

- In Figure 5, H2009 cells are not included in the data set.

→ Because we used the CL1-5 cells as the representative, the data of H2009 was not present.

- In Figure 6c, H2009 cells are not included in the gelatin zymography.

→ Because we used the CL1-5 cells as the representative, the data of H2009 was not present.

- The authors did not identify molecular mechanism between COX Va and MMP proteins although the authors mentioned about the possibility that Bcl-2 would be related in Discussion section. The authors have to prove this possibility with experiment.

→ We have evaluated the expression of Bcl-2 and found it was associated with the expression of COX Va. We have added the data in the manuscript and Fig.7. We hope this can help to improve the mechanistic insight of our current study.

- In Fig.7a, the comparison of COX-Va expression between Normal and Tumor tissue indicate not metastasis but tumorigenesis. Is there any correlation between the over-expression of COX Va and metastasis in the patients who donated the samples?

→ The patient 3, 7 and 8 did have metastasis. However, it is difficult to tell whether there is any correlation between COX Va overexpression and metastasis from these ten representatives. Regarding this concern, we had an analysis from 250 tissue core arrays in Table 2 and demonstrated that COX Va overexpression was correlated positively to metastasis.
To Reviewer 2:

Major Compulsory Revisions:

1. Figure 2.
   - In the methods section, the authors state that the invasion assay was incubated for 18 hours but the figure is characterized with 6 hours. How long was the time of incubation in the invasion assay? If the time of incubation was 6 hours, the invasive potential would be significantly higher than the migration. This interesting fact should be clarified and discussed.
   
   The differences in the migration and invasion assays are highly significant, a statistical analysis for this figure but also for figure 1 would be useful.
   → We thank the reviewer’s carefulness. The “6 hours” in the figure was in fact a typo. We apologize for such a mistake and have corrected the picture. Meanwhile, we have added the statistical analysis in the figures and figure legends.

2. Figure 4A.
   - How many experiments were performed? A quantification of the amount of COX Va according to #-actin would be useful.
   → We thank the reviewer’s great opinion. We performed triplicate in each study and a quantification evaluation was also added. We have provided the data in Figure 4 and figure legends.

3. Figure 4B.
   - A loading control is missing. The molecular weight of COX Va is not mentioned.
   → A loading control was added, and the molecular weight of COX Va was also added.

4. Figure 5A.
   - As for figure 4A, a quantification of COX Va according to #-actin would be interesting. How many experiments were performed? Which construct is transfected with the scramble control - control or vector? Please, clarify this in the figure legend.
   → We thank the reviewer’s great opinion again. We performed triplicate in each study and a quantification evaluation was also added. We have provided the data in Figure 5 and figure legends. The scramble control was in fact the “vector”. We have corrected the possibly misleading name in both the manuscript and the figures.

5. Figure 5Band C.
   - The scaling of the bar diagrams is not adjusted correctly. Also here, which construct
represents the scramble control?
→ We thank the reviewer’s carefulness. We have corrected the scaling error. Again, the scramble control was in fact the “vector”. We have corrected the possibly misleading name in both the manuscript and the figures.

6. Figure 6A.
- A control as used for figure 2A and 5A is missing. Also here, a quantification would be more convincing. As mentioned for figure 5, which construct represents the scramble control?
→ A control and a quantification plot have been added. Similarly, the “vector” was changed to “scramble” in the figure.

Minor Essential Revisions:
- The second part of the Background should be shortened, especially the last paragraph which belongs rather to the results or discussion section.
→ We have rewritten the second paragraph of the Background section. Thanks for the reviewer’s great comment.

- Reference (1) clearly states that lung cancer is the leading cause of cancer-related death among males, as opposed to breast cancer in females.
→ We thank the reviewer’s carefulness. We have corrected it as the leading cause of male cancer-related death.

- It seems that reference (2) has no relation to the authors’ statement “Despite application of multimodal treatments, the overall survival of NSCLC patients remains poor” (Background, page 5, first paragraph).
→ We considered this citation was improper and have changed it.

- The authors should clarify which antibody they used for detection of cytochrome c oxidase Va in the methods section under the category “Western Blot” on page 12/13. From which company is the antibody?
→ Thanks for the reviewer’s carefulness. The antibody was described in the immunocytochemical staining part but neglected in the Western Blot part. We have added the information in the manuscript.

- The authors used a scramble control as a control for their knockdown experiments. The sequence of this scramble control is missing. The authors should include this missing sequence in the corresponding methods section (COX Va Gene Knockdown,
We thank again for the reviewer’s remind. We have added the sequence in the method section.