Author Response 1

Reviewer: 1

Comments to the Author

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The authors examined the functional role of CASC2, a long non-coding RNA down-regulated in sera of COPD patients, in human bronchial epithelial cell line 16HBE. Although the manuscript seems to be well-written, it also contains some issues as follows:

Reply: Thank you for your review, and thanks for your valuable comments. Those comments are helpful to improve the article. We revised the full manuscript carefully according to the comments.

1. Authors demonstrated that CASC2 was down-regulated "in sera" of COPD patients (Fig. 1A). However, the functional role of CASC2 was examined "in 16HBE". Why? Authors should show data that serum CASC2 is derived from patients' epithelial cells. Showing data that demonstrate an down-regulation of CASC2 in epithelial cells of the patients would also strengthen the manuscript. Alternatively, at least, a down-regulation of CASC2 secreted in culture medium should be shown in the 16HBE experiments. Similarly, the cell origin of serum miR-18a in COPD patients is unclear.

Reply: Thank you for your valuable comments. According to the advice, the level of CASC2 and miR-18a was detected in the culture medium of 16HBE cells, and the results were shown in the revised Figure 2. In addition, as you mentioned, we did not focus on the cell origin of serum miR-18a in COPD patients and the quantitative relations between alterations in intracellular miRNA concentrations and serum miRNA levels are not known, such limitation was discussed in the Discussion section, and it will be explored in the future study.

2. Authors should clearly specify which individual miRNA they analyzed: miR-18a-5p or miR-18a-3p. This information is very important when comparing the presented data with other studies' results. Also, it must be used overall in the manuscript. In addition, detailed information of miR mimic and inhibitor used should be provided (e.g., origin, sequences, etc.)

Reply: Thanks for your review and valuable comments. We have confirmed that it is miR-18a-5p, and the full text was revised as suggested. In addition, information of miR-18a-5p were added according to the advice.

3. (Page 15, line 5) Down-regulation of IGF1 corelated with the CASC2 level in COPD patients should be shown by the authors.

Reply: Thanks for your review. According to the advice, the correlation between serum IGF1 and CASC2 levels were analyzed, and the results were recorded in the revised Figure 6G.

4. Appropriate control experiments are missing. For example, in Figs. 4 and 5, the effects of miR-18a mimic and inhibitor should also be examined in the CSE-untreated cells.

Reply: Thanks for your advice. According to the comments, the effects of miR-18a mimic or inhibitor, even CASC2 were examined in the CSE-untreated cells, and the results were recorded in the revised Figure 2-4, and Figure 6.

5. Mechanism of down-regulation of CASC2 induced by CSE is unclear.

Reply: Thank you for your review on our article. In the current study, the serum level of CASC2 was detected in the clinical serum samples, and the ceRNA regulatory role of CASCA in miR-18a-5p was further explored. The regulatory role of both CASC2 and Mir-18A-5p in 16HBE cell apoptosis and inflammatory response was explored. Furthermore, the target relationship between mIR-18a-5p and IGF1 was proved. As you mentioned, the direct regulatory role of CASC2 with IGF1 was not explored in the current study. It will be considered for further exploration in future studies. Such content was discussed in the Discussion section.

6. (Fig. 2E) Mechanism of inhibition of inflammatory cytokine expression by CASC2 is unclear. Does IGF1 also participate in it?

Reply: Thanks for your attention. As mentioned, CASC2 upregulation leads to the inhibition of inflammatory cytokines. Then the following experiments indicated that miR-18a-5p mimic transfection reversed the inhibitory effect of CASC2 on inflammatory cytokines, and IGF1 was proved to be the target gene of miR-18a-5p. Therefore, we speculated that overexpression of CASC2 might inhibit the bronchial epithelial cell apoptosis and inflammatory via targeting miR-18a/IGF1 axis. In addition, association between the serum levels of CASC2 and IGF1 was analyzed in the revised Figure 6G, reflecting the regulatory role of IGF1 and CASC2 and supporting our conclusion about involvement of CASC2/ miR-18a/IGF1 axis in the disease mechanism. But the direct regulatory role between CASC2 and IGF1 in vitro was not explored in the present study, which should be further verified in future studies. We discussed the limitation in the Discussion section.

7. (Figs. 2B and 4A) How did the authors distinguish endogenous CASC2 and miR-18a from exogenously applied them?

Reply: Thanks for your attention. As shown in Figure 2B and 4A, the exogenous CASC2 and miR-18a was transfected into cells using lipofectamine 3000, and to evaluate the transfection efficiency, the CSE group was established as the negative control group. The expression levels of CASC2 and miR-18a in CSE group reflected the endogenous expression levels. In addition, according to the above comments, the effects of miR-18a mimic and inhibitor are also examined in the CSE-untreated cells (as shown in the revised Figure 4), it more directly reflects the transfection efficiency.

8. (Fig. 4A, CSE+CASC2 group) The result does NOT support authors' idea that "CASC2 functions as a ceRNA of miR-18a".

Reply: Thank you for your review. As mentioned, we checked the Figure 4A, it's exactly what you said that the results are somewhat contradictory. We repeated the experiments and the results

were recorded in the revised Figure 4, which supported our idea that “CASC2 functions as a ceRNA of miR-18a”. Maybe some error was made in the collation of the initial experimental results, and the we checked the original data of the Figure 4A, it is found that, to better shown the expression change of miR-18a in different groups, data in CSE+miR-NC group was normalized to 1, and levels in other groups were also normalized. But we noted that the original data (no normalized) of CSE+CASC2 group was used when generate the figure results. We are sorry for our carelessness, and thank you for your kind reminder. The results were corrected in the revised Figure 5.

Minor:

1. It might be hard to distinguish respective groups among the columns in Figs (e.g., Fig. 4D). Color Figs. would be helpful for readers.

Reply: Thanks for your reminding. The labels of the Figures were revised according to the comments.

2. Check spelling and English grammar well.

Reply: Thanks for your review. We checked and revised the full text carefully to avoid the grammar mistakes.

Reviewer: 2

Comments to the Author

In this MS authors were tried to elucidate the role of lncRNA cancer susceptibility candidate 2 (CASC2) and their possible molecular targets like miR-18/IG1 in the Chronic obstructive pulmonary disease (COPD) model. For that, they used serum of subjects as well as cigarette smoke extract (CSE)-induced 16HBE cells as a model. The following comments/suggestions will improve the quality of this work.

Reply: Thank you for your review, and thanks for your advice on our article. Based on the advice, we revised the full manuscript carefully. We hope the revision can meet the approval.

Major Comments:

1. Authors used only CASC2 expression level in serum, what about the CASC2 expression level in lung tissues. We can use serum level for non-invasive diagnostic purposes, but the tissue expression level is critical for COPD phenotype. Authors should show tissue CASC2 level in either qPCR or histochemistry method.

Reply: Thanks for your attention. We agree with you that it can improve the article to detect the CASC2 expression level in lung tissues, but we are sorry for that during the present study, we just collect the serum samples of the study subjects. In future studies, tissues samples collection will be considered. We discussed the limitation in the Discussion section.

2. Are all subjects under the same GOLD stage or from different GOLD stages. This will indicate that weather CASC2 associate with the early onset of COPD or at later stages.

Reply: Thanks for your attention. According to the comments, GOLD stage of the patients were added in the methods section. and the level of CASC2 in different groups were analyzed and shown in the revised Figure 1C.

3. What is the source of research-grade cigarettes used in this study.

Reply: Thanks for your attention, the mentioned information was added.

4. What is the expression level of CASC2 in overexpressed condition (Fig.2B) without CSE

Reply: Thank you for your attention, the mentioned results were shown in the revised Figure 2.

5. In this study authors focused only inflammation as a phenotype of COPD, what about others, and how it's associated with lncRNA CASC2.

Reply: Thank you for your attention. As previous studies reported, CASC2 plays an important role in inflammatory response, considering the crucial role of inflammatory in the pathological mechamnism of COPD, we explored the effect of CASC2 in inflammatory response in vivo. As you mentioned, COPD encompasses a variety of clinical and pathologic phenotypes ranging from airway inflammation (chronic bronchitis) to destruction of lung tissue (emphysema) and remodeling of the small airways. But in the current study, we just focus on the inflammatory condition of COPD, association of CASC2 with other phenotypes were not included in the current study. That might be limitation of the study, and we discussed it in the Discussion section. and it will be considered for further exploration.

6. In the methods section authors mentioned both student t-test and ANOVA were used in this study, but results/fig legends did not mention where they used t-test / ANOVA.

Reply: Thank you for your review. According to the advice, the statistical methods were added in the Figure legends.

Minor comments:

1. On page 10 lines 8-10 there was no clarity in the sentence.

2. On page 11 line 25 authors used ‘CSE-induced cell viability inhibition', authors should correct this sentence.

3. The expansion of CASC2 should mention in the abstract rather than discussion.

Reply: Thank you for your careful review of the article. According to the comments, the mentioned sentence was revised, and the full name of CASC2 was added in the abstract section.