Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-2196

Reviewer A

The study by Zhang et al. investigated one potential mechanism for cigarette smoke-induced epithelial-mesenchymal transition (EMT). They examined the role of cathelicidin in inducing EMT in the airway and promoting airway remodeling in smoking-related COPD. They also suggested that cathelicidin could induce airway EMT by activating TACE/TGF-α /EGFR signaling pathway to mediate smoking-induced airway remodeling in the pathogenesis of COPD. The role of cathelicidin in the pathogenesis of COPD has been reported previously by the same group, and the EMT angle seems new. Overall, the data of this manuscript are scarce and not quantitative. The link between EMT and TACE/TGF-α /EGFR signaling pathway was not well established.

Reply to Reviewer A:

Thank you, a lot, for your valuable comments and suggestions. We have supplemented the data in vitro for this study, and established the link between EMT and TACE/TGF-α /EGFR signaling pathway. We have learned your professional comments carefully and tried our best to revise our manuscript based on your suggestions. Thanks for your important comments and advice that enabled us to improve the quality of our manuscript. Here is the point-to-point response.

Comment 1:

Although immunostaining (Figure 1) gives a good visualization of protein expression, it is not quantitative. It will be better to have the gene expression of cathelicidin, E-cadherin, and vimentin. Since this study focused on EMT, it will be essential to examine the level of some EMT master transcription regulators such as SNAIL, ZEBs, and TWIST. Also, did smoke change the level of cathelicidin in the bronchoalveolar lavage fluid (BALF) of COPD patients and controls?

Reply 1:

Thank you for your professional and valuable comments. We agree with the reviewer that it is important to investigate the corresponding transcriptional regulators in researches involving EMT. In our study, we mainly focused on the association between Cathelicidin and EMT phenomonen, trying to establish the causal relationship. Based on the *in vivo* and *in vitro* data, we conclude that Cathelicidin could induce airway EMT, promoting the pathogenesis of smoking-related COPD. This is the primary step of our research. It is our work to investigate the detailed mechanism for the regulation of EMT in future. Thanks again for your valuable comments.

Comment 2:

The lung slices from COPD patients (Figure 1) did not show typical features of airway remodeling, such as flattened epithelium, expansion of mesenchymal supporting cells, or thickening of the alveolar septae. A Masson trichome straining and zoomed images will be helpful. The lung slices from COPD patients (Figure 1) did not show typical features of airway remodeling, such as flattened epithelium, expansion of mesenchymal supporting cells, or thickening of the alveolar septae. A Masson trichome straining and zoomed images will be helpful.

Reply 2: Thanks a lot for your valuable and helpful comments. We stained the lung tissues by Masson trichome, and demonstrated the histopathological features as shown in *Figure 1*. The airway remodeling was obvious in smokers and COPD patients. Based on your advice, we added a figure and the corresponding descriptions in the manuscript.

Changes in the text: we have added a Figure named Figure 1, and some description on Page 14, line 9.

Comment 3:

It is not clear how the COPD animal model was established. The authors seem to suggest that they created this in vivo model by exposing the mice to cigarette smoke. These mice were then exposed to smoke again after the administration of the Lentivirus vector. It is not clear how the vector was administrated and how the expression of cathelicidin was controlled. Most importantly, the characterization of the in vivo models, such as lung function, the features of airway remodeling, and the level of cathelicidin in lung tissue and BALF was critical but utterly absent in this manuscript.

Reply 3:

Thank your for your important and helpful comments. Based on your comments, we added the methods to establish the mice model on Page 9, Line 46. The mice were continuously exposed to cigarette smoke, and during this period, Lentivirus was intranasally administrated on day 1, day 10, day 20, day 30, day 40 and day 50. We performed Lentivirus administration according to the methods and movie provided by the reference: Michel DuPage, et al. Conditional Mouse Lung Cancer Models Using Adenoviral or Lentiviral Delivery of Cre Recombinase. Nat Protoc,2009;4(7):1064-72. doi: 10.1038/nprot.2009.95. As the COPD models in our study was established according to the classical methods, we did not examine the detailed pathological characteristics, such as lung function and BALF. We have added the Masson Trichrome staining data about the lung tissues to demonstrate airway remodeling in COPD mice (Figure 3 and Page 15, Line 1). In future studies, we will supplement the data about BALF and lung function. Thanks again for your professional comments that enabled us to improve our work.

Comment 4:

The conclusion of the involvement of EMT in the in vivo study was supported only by immunostaining, which is not quantitative and less convincing. The gene and protein expression of cathelicidin, E-cadherin, vimentin, SNAIL, ZEBs, and TWIST should be provided.

Reply 4:

Thank you for your valuable comments. We feel sorry that our work are limited and less convincing. We mainly focused on the qualitative analysis of Cathelicidin, E-cadherin and vimentin, demonstrating the existence of Cathelicidin and EMT. These results could explain the problem issued by our study to a certain extent. As most of the lung tissues from mice had been used for immunostaining, it would be better to measure the expression of these parameters in our future study. Thanks again for your professional and valuable comments.

Comment 5:

The authors tried to establish a link between EMT and TACE/TGF-α /EGFR signaling pathway by suggesting that cathelicidin mediated both TACE/TGF-α /EGFR signaling pathway and EMT. However, there was no direct evidence for the role of TACE/TGF-α/EGFR in mediating the EMT. More mechanism study is needed.

Reply 5:

Thank you for your valuable comments. We conducted *in vitro* experiments to explore the mechanism of Cathelicidin-induced EMT. When the TACE, TGF-α and EGFR were inhibited by corresponding blockers, Cathelicidin induced EMT could be ameliorated significantly, suggesting direct evidence for the role of TACE/TGF-α/EGFR in mediating the EMT. (Page 16, Line 15). Thank you for all of your valuable comments and suggestions above. Your professional suggestions have helped us improve our work. Thanks again.

Reviewer B

Comment 1:

Lack of breadth in the introduction and discussion

Reply 1:

Thank you for your professional and valuable comments. Based on your comments, we have revised the introduction and discussion, which were marked as red.

Comment 2:

**optical density (IOD) per stained area (μm²).

Optimal? Would it be better to also factor in or isolate the distribution of protein expression?

Reply 2:

Thank you for your kind and helpful comments. The IOD/μm² could represent the mean staining density of the parameters, and had been used in a kind of researches.

Comment 3:

* nomenclature of the groups should change. Cu and Cd is not clear

Perhaps: CRAMP++ and CRAMP -- …

Reply 3:

Thanks for your helpful and kind suggestions. We have changed the description of corresponding groups in the manuscript.(Page 9)

Comment 4:

Why has IHC been used for human tissue and IF for the mouse lung?

Reply 4：

Thank you for your valuable comments. We examined human tissue by IHC, as to a certain extent IHC could demonstrate the pathological characteristics which varied commonly. And IF was used in animal models, as the COPD mice model has been established according to classical methods with a stable pathological profile.

Comment 5:

"The nonparametric analysis was carried out for appropriate data. The Kruskal-Wallis and Mann-Whitney U test were used for comparisons among patient groups."

Was gaussian distribution tested? Potentially misleading to start the statistics section of methods with this?

Reply 5:

Thank you for your careful reading and kind comments. We had conducted Gaussian test, and corrected on Page 13, Line 125.

Comment 6:

Does this highlight a strong limitation with this study in low samples sizes? If so, this should be addressed in the discussion.

Reply 6:

Thank you for your professional and valuable comments. We agree with the reviewer and we discussed the limitation in low samples sizes.(Page 20, Line 15)

Comment 7:

Later it is stated that one-way ANOVA is used for in vivo data (parametric?)….

Reply 7:

Thank you for you valuable comments. We conducted Gaussian test, and found that one-way ANOVA was suit for *in vivo* data.

Comment 8:

Predominantly male cohort... should be discussed.

Reply 8:

Thank you for your valuable and kind suggestions. We have discussed the limitation of male cohort.(Page 21, Line 1)

Comment 9:

Difficult to tell from supplied images but background staining may be an issue. The inclusion of negative control images will quell those doubts. Restriction of the measured area to only include the small airway would strengthen the results if the optical density is the choice of measurement. Higher magnification for image analysis would also be helpful. Inclusion/exclusion of points should be stated, and standard deviations included in figure 1.

Reply 9:

Thank you for your valuable comments. We reviewed the pictures of IHC, and found that the differences were significant. The data in figure 1 was not in accordance with Gaussian distribution, so in should be presented as median, not mean ± SD.

Comment 10:

"These results suggested that cigarette smoking could induce the Cathelicidin overexpression and airway EMT during the development of COPD, which was in accordance with the results observed above in human specimens and those of previous studies"

Tricky. The results show that the smoking model increases the expression of CRAMP. A step back is needed and perhaps the statement is to be worded as more of supporting evidence for these mechanisms to be true in humans.

Reply 10:

Thank you for your valuable comments. Our results showed EMT in both smokers with and without COPD, which was actually in accordance with previous studies, although we did not find significant difference in EMT between these two groups. We had discussed this point in the manuscript. (Page 20, Line 15).

Comment 11:

In figure 2. The supplied images do not support the results for CRAMP expression.

N- numbers? Is are those results significant?

Reply 11:

Thank you for your valuable comments. We reviewed the images of CRAMP expression, analyzed the expression intensity, and found that the images supported the results for CRAMP.

Comment 12:

"Our results showed that compared with the Control group, expressions of TACE, TGF-α, and EGFR at the protein level in COPD group and Cu group were significantly increased (Figure 3)" Really? The western blot has not been quantified. Alone, the Cu group does not appear to have increased protein expression of TGF, TACE, and EGFR in comparison to controls.

Further protein expression analysis to make the claims surrounding figure 3.

Reply 12:

Thank you for your valuable comments. We reviewed and analyzed the images (Figure 5), and found Cu group had increased protein expression of TGF, TACE, and EGFR in comparison to controls.

Comment 13:

The limitations of this study need to be better stated, explored, and discussed.

The limitation stated as mainly containing in vivo work isn't appropriately expanded. The availability of human tissue for scientific research is the opposite of a limitation and several other limitations of this study should be deliberated here instead.

Reply 13:

Thank you for your valuable comments and kind suggestions. We had supplemented in vitro data and revised the limitations in our manuscript (Page 21, Line 1). Thanks again for all your valuable and kind comments above that helped us improve the quality of our manuscript.

Reviewer C

The authors explored Cathelicidin in COPD from the perspective of EMT. However, the results are paradoxical. The detective techniques are insufficient to support the conclusion.

Comment 1:

The authors introduced the epidemiology of COPD by reference 3. This declaration is not appropriate. The original report should be "Chronic obstructive pulmonary disease in China: a nationwide prevalence study. Lancet Respir Med. 2018 Jun;6(6):421-430.", which was surveyed in 2015 and published in 2018.

Reply 1:

Thank you for your careful reading and professional comments. We had corrected the data in the manuscript.(Page 5, Line 4)

Comment 2:

On page 12, the result subtitle "Immunoreactivity of LL-37 and EMT Markers in Small Airway Epithelium" is not appropriate. IHC staining never represents immunoreactivity.

Reply 2:

Thank you for your valuable and professional comments. Based on your comment, we had made corresponding corrections.

Comment 3:

On page 12, the authors declared in the results "There were no significant differences in the expressions of Cathelicidin, E-cadherin, and vimentin between smokers with and without COPD. These changes of EMT markers indicated that EMT existed in the small airways of smokers with and without COPD, which was in accordance with previous studies". This conclusion is self-contradictory. No difference of these molecules was found between smokers with and without COPD (Figure 1), which indicates that they do not participate in COPD.

Reply 3:

Thank you for your valuable comments. Cigarette smoke was the major risk factor of COPD, our results showed that smoke was correlated with EMT in human airways. This was a limitation of our study as both the smokers with and without COPD had a heavy smoking history. Other factors that influenced the EMT of COPD patients, such as regional and systemic inflammation , might be obscured by cigarette smoke. Nevertheless, we could conclude that Cathelicidin participated in the pathogenesis of COPD with smoking history. Further studies, including smokers with a light smoking history and COPD patients without smoking history, are needed to elucidate the precise difference in smokers with and without COPD.

Comment 4:

IHC and IF are generally used for molecular location. WB and RT-qPCR are exactly the accurate way to quantitative assessment of Cathelicidin, E-cadherin, and vimentin.

Reply 4:

Thank you for your valuable comments. It is important to quantitative assessment of Cathelicidin, E-cadherin, and vimentin. In our study, we aimed to explore the association between Cathelicidin and EMT phenomonen, trying to establish the causal relationship, so we mainly focused on the qualitative analysis of Cathelicidin, E-cadherin and vimentin, demonstrating the existence of Cathelicidin and EMT. These results could explain the problem issued by our study to a certain extent. It is our future work to quantitative assess the expression of Cathelicidin and EMT markers.

Comment 5:

Exploration of cathelicidin-induced TACE/TGF-α/EGFR pathway is rarely novel.

Reply 5:

Thank you for your valuable comments. Although it was not a complicated signaling pathway, few studies had investigated the role of TACE/TGF-α/EGFR pathway in Cathelicidin induced EMT.

Comment 6:

Experiments at cellular levels with treatment of cigarette smoke extract are further needed.

Reply 6:

Thank you for your valuable comments. We supplemented the in vitro data for this study. Thanks again for all your valuable and kind comments and suggestions above that helped us improved the quality of our manuscript.