Author's response to reviews

Title: Protocadherin-1 is a glucocorticoid-responsive critical regulator of airway epithelial barrier function

Authors:

Yutaka Kozu (blackflag@onyx.ocn.ne.jp)
Yasuhiro Gon (gon.yasuhiro@nihon-u.ac.jp)
Shuichiro Maruoka (maruoka.shuichiro@nihon-u.ac.jp)
Kuroda Kazumichi (kuroda.kazumichi@nihon-u.ac.jp)
Akiko Sekiyama (akiko.wish@key.ocn.ne.jp)
Hiroyuki Kishi (kishi.hiroyuki@nihon-u.ac.jp)
Yasuyuki Nomura (nomusan@siren.ocn.ne.jp)
Minoru Ikeda (ikeda.minoru@nihon-u.ac.jp)
Shu Hashimoto (shuh@med.nihon-u.ac.jp)

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Author's response to reviews: see over
We wish to express our appreciation to the reviewers for his or her insightful comments, which have helped us significantly improve the paper.

Reviewer #1 (Remarks to the Author):

With regard to the reviewer’s comment on “A major drawback of this study is that only immortalized cell lines and tumor cell lines were used to study the function of PCDH1. The extent to which findings on epithelial barrier functions obtained using such cells can be transferred to primary epithelial cells is unknown. This limitation should be discussed in detail.”

Response; we appreciate the reviewer’s suggestion. One of the limitations of our study is that we did not study the effect of PCDH1 knockdown on primary cultured epithelial cells. Primary airway epithelial cell needs more than 7 days to make tight junction barrier. We don’t have now the method to transfect efficiently miRNAs into primary airway epithelial cells and keep it for a certain period. So, we could not examine that on primary airway epithelial cells. Therefore, we added the description, “In the present study, we did not study the effect of PCDH1 knockdown on primary cultured epithelial cells, because we could transfec efficiently miRNAs into primary airway epithelial cells and keep it for a certain period. So, it is one of the limitations of our study.”, page 13, line 20 of the revised manuscript.

With regard to the reviewer’s comment on “Page 11: Lung tissue samples from asthmatics and controls were obtained from patients undergoing surgery for pneumothorax or lung cancer. The authors should discuss whether and how this could have affected the outcome of the study. Furthermore, it is important to characterize the asthma patients in this study with respect to the severity of their disease.”

Response; we appreciate the reviewer’s comment. It is very difficult to obtain the lung sample from the asthmatic patients. Therefore, it is one of the limitations in our study. We used several source of lung section including 2 autopsy samples obtained the fatal asthmatic patients. In common, all patients were relatively severe asthmatic patients with airflow limitation. Recently, pathogenesis of asthma is recognized to be heterogenous and complexities. Although we couldn't clarify how the PCDH1 is involve in the pathophysiology of asthma, we showed that downregulation of PCDH1 would be associated with progression of disease severity of asthma. To explain our study
limitation, we insert the sentence “We used several source of lung section including 2 autopsy samples obtained the fatal asthmatic patients. In common, all patients were relatively severe asthmatic patients with airflow limitation. Recently, pathogenesis of asthma is recognized to be heterogenous and complexities. Although we couldn't clarify how the PCDH1 is involve in the pathophysiology of asthma, we showed that downregulation of PCDH1 would be associated with progression of disease severity of asthma.” in the results of the revised manuscript (page 14, line 23).

*With regard to the reviewer’s comment on “Please indicate how the authors determined that the epithelial cultures were indeed polarized as written by the authors.”*

Response; we checked the polarity of epithelial cells measured by transepithelial electrical resistance each experiments. We also checked the apical-basal polarity of epithelial cells by the localization of E-cadherin and ZO-1 in figure 4. The apical distribution of TJ and AJ proteins are most important feature of polarity of epithelial cells.

*With regard to the reviewer’s comment on “Western blotting and the antibody used are not described in the main manuscript. Yet these are important issues that should be part of the main manuscript.”*

Response; we appreciate the reviewer’s comment. According to the reviewer’s suggestion, we moved the materials and methods of western blotting to the main manuscript from the supplemental information.

*With regard to the reviewer’s comment on “what evidence do the authors have that the cells used in the siRNA knockdown experiments were differentiated as claimed by the authors in line 212.”*

Response; in general, it is thought that the apical-basal polarity is the most important feature of epithelial differentiation. We carefully evaluated the status of the apical-basal polarity by transepithelial electrical resistance and the distribution of E-cadherin and ZO-1 protein.
With regard to the reviewer’s comment on “Since the authors did not assess airway hyperresponsiveness and/or reversibility of the asthma patients and controls, it is unclear how asthma was diagnosed.”

Response; we appreciate the reviewer’s comment. According to the reviewer’s suggestion. We usually use airway hyperresponsiveness and/or reversibility for the diagnosis of bronchial asthma, but not all. In the present study, all patients were diagnosed by the criteria of the Global Initiative for Asthma guidelines.

With regard to the reviewer’s comment on “What was the control of siRNA PCDH1? Was it a scrambled form?”

Response; we appreciate the reviewer’s comment. We used the Stealth RNAi negative control duplexes (invitrogen) as a negative control. To adjust the GC contents levels of PCDH1 siRNA, we used the medium GC Duplex (cat. 12935-112). We put the catalog number of siCtlRNA in the materials and methods (page 6 line 6).

With regard to the reviewer’s comment on “Were the samples boiled after addition of sample buffer?”

Response; we appreciate the reviewer’s comment. To explain clearly, we changed the above sentence to “The samples for PAGE analysis were mixed with 4× XT sample buffer (Bio-Rad, Hercules, CA) and boiled for 4 min., and separated on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA)” (page 7, line 20).

With regard to the reviewer’s comment on “This supplemental figure was most likely damaged during pdf conversion, but could be evaluated to some extent.”

Response; we appreciate the reviewer’s comment. We are carefully re-uplooaded the supplemental figures. Thanks for the reminder.

Reviewer #2
With regard to the reviewer’s comment on “In order to infer mechanism, we need to be sure the PCDH1 knock-down does not interfere with expression of related family members. Consequently, please provide statistical analysis of the Western blots of PCDH1 and E-cadherin levels following PCDH1 siRNA transfection (Fig 2C and Supplementary Figure 3).”

Response; we appreciate the reviewer’s comment. We showed statistical analysis of Western blots of PCDH1 and E-cadherin levels following PCDH1 siRNA transfection. As shown in figure 2C and supplement 3. The knockdown of PCDH1 by its siRNA transfection did not influence E-cadherin expression. We revised the figures and figure legend of figure 2C and supplement 3.

With regard to the reviewer’s comment on “The legend to Figure 6 does not correspond to the panels shown, and is confusing. Please define what “EDC” stands for (Fig. 6D). The distinction between “inflamed” and “non-inflamed” sections of lung tissues is important but seems arbitrary: please clarify how this was done objectively. I was not clear if the authors observed a difference in PKDH1 expression between lung tissue samples from asthmatic vs. healthy control subjects. Given variability in TJ/AJ expression, I would be surprised if there were substantial differences between groups given the relatively small numbers of subjects studied (9 subjects per group), but this should be stated clearly.”.

Response; we appreciate the reviewer’s suggestion. According to the reviewer’s suggestion, we corrected Figure 6 legend. We explained full spell of “EDC”. To make sure the criteria of “inflamed” and “non-inflamed” sections of lung tissues, we have inserted the sentence “the noninflamed region (NR), where there are few infiltrated inflammatory cells and CECs are histologically intact. We also examined inflamed regions (IR) where inflammatory cells such as eosinophils and lymphocytes had infiltrated the submucosa and where histology indicated that the CECs had sustained damage such as partially shed epithelium or separation of cell junctions.” in the materials and methods of the revised manuscript. (page 8, line 5). In the present study, we could not observe any difference in the expression level of PCDH1 between the patients and controls. As the reviewer’s pointed out, the description of this result was unclear. We have inserted the sentence “There are no differences on the expression levels of PCDH1 between normal mucosa in control and NR in CRS. …There are no differences on the expression levels of PCDH1 between normal mucosa in control and NR in asthma” in
the results of the revised manuscript (page12, line6, and page12, line12). We could not evaluate the difference of the expression AJ or TJ proteins in the nasal or lung tissues because of small number of the sample number. We have inserted the sentence “We could not compare the expression levels of PCDH1 and other TJs / AJs proteins” in the section of discussion in the revised manuscript (page14, line17).

With regard to the reviewer’s comment on “Since PCDH1 knock-down apparently disturbs other junctional structures, it remains to be seen whether this molecule itself contributes to barrier integrity directly (e.g. via cell-cell homotypic interactions) or indirectly (e.g. via stabilizing other AJ or TJ components). Please make this clear in the Discussion.”

Response; we appreciate the reviewer’s comment. We inserted the sentence “This suggests that PCDH1 facilitates E-cad assembly, and its loss would inhibit TJ formation through direct or indirect mechanism” in the section of the discussion in the revised manuscript (page13, line17).

With regard to the reviewer’s comment on “Another area of potential interest is the role of PCDH1 in respiratory virus mediated junctional dysfunction. Some studies alluding to the role of viruses in barrier dysfunction would seem indicated in the Discussion (e.g. reviewed in PMID 25085341)”

Response; we appreciate the reviewer’s comment. We referred a review as the reference number 24.

With regard to the reviewer’s comment on “1. Please confirm that the difference between 124 and 122 eosinophils (/mcl) was statistically significant (p<0.03) in Table 1. 2. Supplemental Figure 2A was jumbled after downloading.”

Response; thank you for pointing that out. It was an error caused by a typing mistake. The correct numbers of eosinophils are 122 in non-CRS and 345 in CRS. The difference is a statistically significant. We corrected these numbers in the Table 1.