Author's response to reviews

**Title:** Clinical significance of mTOR, ZEB1, ROCK1 expression in lung tissues of pulmonary fibrosis patients

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**Author's response to reviews:** see over
Dear Prof. Mark Dransfield,

Thank you for your positive response to our article. We appreciate the feedback and have strove to incorporate that feedback into our article. We used underlined letters for revised sentences in the manuscript.

Reviewers’ comments

< Reviewer 1>

Major Compulsory Revisions:

1) As the authors followed the patients over time, they presented the expression data in the tables as change over FVC. Opposed to using change over time for all patients, it would be very interesting to present all expression data according to severity of disease using FVC, extent of honeycombing by CT scan, DLCO and histologic fibrosis score upon biopsy, as shown for mTOR expression in Figure 2. There is no mention that this analysis was performed for ZEB1 and ROCK1. This information along with the survival curves could further stratify a subset of patients with poor prognosis. By doing this it is possible that ROCK may emerge to be significant marker.

   We examined all the expression data in association with disease severity by using FVC, DLCO, honeycombing, and histologic fibrosis score. However, no correlations were found, except between mTOR and fibrosis score. Our findings have been described in the “Results” section, page 9-10 and added in Figure 3 and 4.

2) The authors mentioned that ANOVA was performed. However, was this performed for
each stain with respect to patient samples? It would actually be of interest to do ANOVA across expression of each marker per patient. This would further determine, if collectively together these markers are important.

Initially, we used ANOVA to analyze the differences in marker expression among the three groups (negative, intermediate, and strong group). However, to highlight the differences, we re-categorized the patients into two groups such that the negative and intermediate groups were combined together to form one group that was compared with the strong group. The two groups were analyzed by Fisher’s exact test and Mann-Whitney Test. To avoid confusion, we deleted the term “ANOVA” in the manuscript on page 8. Furthermore, following your recommendation, we performed statistical analysis for all markers per patient and altered the Tables 2, 3, and 4. We have added the results on pages 9-10.

3) To better appreciate the co-localization of these markers in specific lung tissue, it would be advantageous to co-stain the lung for cell specific markers. Then normalize the staining by immunofluorescence to have a non-bias assessment of the tissue. Also this method would be able to generate a ratio of expression per cell to account for the increase recruitment to the lung tissue.

We could not perform co-staining due to the unavailability of two distinct secondary antibodies for detecting the primary antibodies against epithelial cell markers and the markers of interest (mTOR, ZEB1, ROCK1). However, we tried to quantify expression of markers in alveolar epithelial cells submitting samples for analysis by 2 independent pathologists.

4) In the discussion (example page 10) and throughout the article, the authors indicate that the role of mTOR in human pulmonary fibrosis is being examined. Much of this is speculative and should be rewritten to reflect that the authors postulate the mTOR role based on existing literature.

We revised the discussion to differentiate results of our study from those of the existing literature (page 11).

Minor Essential Revisions:

1) In the abstract, the authors state that either mTOR or ZEB1 expression was associated with better prognosis, however the survival curves show the inverse. This should be corrected.
Thank you for the feedback. We made a mistake, so corrected errors. mTOR or ZEB1 expression was associated with poor prognosis.

2) In the introduction (paragraph 2, second to last sentence) where the authors described the administration of rapamycin to TGF-ainduced pulmonary fibrosis, the reference by Korfhagen et al should be included.

   We inserted reference (reference number 3).

3) Arrows and magnification should be placed on the IHC images.

   We inserted arrows and magnification in Figure and Figure legends.

4) Please indicate the number of control lung tissue samples used in the comparisons.

   We used two control lung tissue samples. We described it “Methods” section on page 6.

< Reviewer 2>

Major Compulsory Revisions

1) The major problem of the study is the population used. In the text the authors mentioned that used 30 cases of pulmonary fibrosis, is necessary that it is explained much better, if used only idiopathic pulmonary fibrosis (IPF) cases or included other type of fibrotic diseases with histologic patter of UIP. In the section “Baseline characteristics of the study” they mentioned that had patients with connective tissue disease. If the author included other type of diseases probably all the clinical statistical analysis will be influenced in function of the type of diseases studied.

   As you pointed out, there were 4 patients with connective tissue disease (CTD). Initially these patients were diagnosed with IPF, but during the follow-up period they presented CTD symptoms. As suggested, the statistical analysis results may be influenced by the inclusion of other types of diseases (CTD-related interstitial lung disease). Therefore, we changed the title from “idiopathic pulmonary fibrosis” to “pulmonary fibrosis patients”. We have described it as limitations in the “Discussion” section on page 13.

2) The criteria used by the authors, American Thoracic Society/European Respiratory Society...
International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias; June 2001, is specific to IPF or the other types of idiopathic diseases and it can’t use if the author included other type of diseases such as vascular lung diseases.

‡ There were no cases with other idiopathic interstitial pneumonia. We only included IPF and CTD-ILD (connective tissue disease related interstitial lung disease). The CTD-ILD case that was included in this study was diagnosed by a rheumatologist after CTD confirmation and it was histologically UIP pattern.

3) How the authors did the analysis of the expression of the different markers including the different areas observed in UIP pattern? It was randomizing?
‡ We randomly selected 10 microscopic fields at 100 × magnification. Each field was individually assessed for scoring. Then, the score for each marker was determined by obtaining the mean score of all the fields. We included these data in the “Methods” section on page 8.

4) There is not a description of the expression observed in the different areas of UIP (normal areas, fibroblast foci areas, honeycombing areas), Was the expression of the alveolar epithelial cells the same in these different areas?
‡ In the UIP lungs, expression of each marker was dominant in the hyperplastic alveolar epithelial cells of the honeycombing area. We added photos in Figure 2 that represents these findings and included the data in the “Results” section on page 9.

Minor Essential Revisions
1) In figures D to F there are other type of cells apparently that expressed the different markers studied. Did the authors do the score on these cells expression, too?
‡ We only scored the marker expression of alveolar epithelial cells and described it in “Methods” section on page 7-8.

2) The authors will can to do a figure panel exemplifying the two principal grades of fibrosis (minimal and severe) studied and the intensity of expression (intermediary and strong) observed with the different markers.
‡ We added Figure 1 that exemplified the grades of fibrosis and intensity of expression.
3) The figure 3 needs the P values. For survival analysis, the authors used only the negative and positive groups, it is true?

We inserted P values in the survival graph (Figure 4). We categorized the 30 lung tissues into negative or positive group for the survival analysis. The positive group included both intermediate and strong expression groups.

4) The authors need included in the different tables the type of statistical analysis used.

We added method of statistical analysis below the each tables.

On behalf of all authors,

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