Author's response to reviews

Title: Folliculin mutations are not associated with severe COPD

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Version: 2 Date: 2 October 2008

Author's response to reviews: see over
Dear Dr. Whitaker,

On behalf of my co-authors, attached please find our point-by-point response to the reviewers and the revised manuscript entitled “Folliculin mutations are not associated with severe COPD” (MS: 1587844992210041). This revision incorporates suggestions offered by the reviewers, and we believe we have addressed all of the criticisms and concerns raised in the review.

We would like to thank all three reviewers for their thorough critique, and hope that our revised manuscript is found to be suitable for publication.

Sincerely,

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**Reviewer 1:**
"Folliculin mutations are causative of a rare inherited dermatologic disorder, Birt-Hogg-Dube’ syndrome with associated lung cysts and spontaneous pneumothorax, and have also been found in the germline of patients with familial spontaneous pneumothorax. Chou et al. present a well defined study to assess the role of folliculin gene variants in the pathogenesis of generalized COPD. They analyzed 152 severe COPD probands for 7 folliculin mutations previously associated with FSP and BHD, and resequenced the folliculin gene in a subset of 41 probands. No patients were found to harbor any of the 7 mutations, and of the 31 variants identified in the resequencing effort, only 2 were predicted to have any functional effect. These two variants and two common non-coding polymorphisms were evaluated in an independent set of cases and controls with no association observed for any of the variants with presence of COPD or emphysema-related phenotypes. The study is well designed using well characterized COPD subjects from a previously reported study, and adequate case and control sample sizes. Methods for sequencing and
genotyping were appropriate and well described. Although nearly 30 folliculin mutations have been reported, the seven folliculin mutations chosen for testing were based on those reported in association with FSP and the common mononucleotide insertion/deletion mutation found in >50% of BHD patients and are, therefore, appropriate choices. The resequencing effort was extremely thorough, including all 14 exons, splice junctions, 5'UTR and 50 bases into intronic regions, and therefore, most likely detected all variants among the 41 probands. The careful re-examination of the two non-synonymous folliculin variants in an additional cohort of emphysema subjects and controls confirmed no association with COPD underscoring differences in molecular pathogenesis between FSP and early-onset COPD. The limitations of the study were adequately discussed by the authors, including 1) the fact that rare variants may be limited to certain ethnic groups and these subjects were mainly Caucasian, 2) the fact that not all folliculin mutations identified to date were screened in this study, and 3) the severe COPD sample size was modest although similar to size of BHD cohorts reported."

Response: We appreciate Reviewer 1’s comments.

"Minor Essential Revisions:
1) Table S1 contains important data to support the conclusions of the paper and should be included in the main manuscript not supplemental files."
Response: We have moved Table S1 into the body of the main manuscript as Table 4, page 20.

"2) Table 2 should include the literature reference for each of the mutations listed to document their discovery in previous work and enable the reader to evaluate genotype-phenotype associations."
Response. We have updated Table 2 to include literature references for each of the folliculin mutations, to document their initial and subsequent discoveries and descriptions of associated phenotypes.

"3) Title of Table 2 should be corrected for capitalization, i.e., Boston and COPD."
Response: We have corrected the capitalization in Table 2.

"4) Legend in Table 3 should state “Referenced to chromosome 17 “ not 7."
Response. We apologize for this error, which we have corrected.

"5) Figure 1 and 2 labels appear in the center of the figures not in the upper left or right corner and should be corrected."
Response: We have moved the labels in Figure 1 and 2 to the side as requested.
Reviewer 2:
"In this paper Dr Cho and colleagues have investigated to potential role of the folliculin gene as genetic determinant for severe COPD. The authors investigated 7 mutations, previously reported in Birt-Hogg-Dubè syndrome or in familial pneumothorax, in 152 patients with severe COPD; none of these mutations was detected in the severe COPD cohort. After resequencing of the gene in 41 COPD subjects, 4 more variants were investigated in 345 subjects from the NETT cohort and 420 smokers from the NAS. Non association was detected. The folliculin gene is a good candidate for emphysema, and the authors have performed a high level genetic investigation in a well defined population with COPD/emphysema."
Response: We appreciate reviewer 2's comments.

"Minor essential revisions
1- One possible extension of the investigation would be to analyse the association with sub-phenotypes of the COPD cohorts, if available, such as bullous emphysema and/or pneumothorax occurrence"
Response: We agree that an examination of these subphenotypes in the COPD cohorts would be interesting. Unfortunately, details on pneumothorax occurrence are unavailable; furthermore, in the Boston Early-Onset COPD Study, details about the characteristics of emphysema are unavailable, and in the National Emphysema Treatment Trial, subjects were specifically excluded if they had pre-existing giant bullae

"2- The authors should explain the reasons why they have selected the 4 variants among the 31 identified in the resequencing experiments"
Response: We have clarified the selection of these variants on page 6 as follows (emphasis added) "Our resequencing results suggest that COPD is not caused by rare, severely deleterious folliculin variation. However, several more common variants were identified that mapped to the folliculin transcript, raising the possibility that folliculin variants with more subtle functional impact may contribute to COPD pathogenesis. To assess this possibility, we genotyped four variants that mapped to the folliculin transcript.. the two nonsynonymous variants (rs3744124 and rs41419545) and two common 5' UTR variants (rs1708629 and rs1736209)"

"3- Did the authors investigate the 4 novel variants in the complete cohort of EOCOPD too ?"
Response: We did not investigate these 4 variants in the complete family based cohort of the Boston Early-Onset COPD Study.

"4- Perhaps the table summarising the results should be moved from the supplemental material to the text"
Response: We agree, and have moved table S1 in the supplementary material to table 4 in the text.

"5- ERJ has in his online section a paper in press from a Swiss group, which associated a novel folliculin gene variant (which is probably included in the series of those detected by Dr Cho) with spontaneous pneumothorax (paper posted on
June 25). If the authors have access to this ERJ section, it would be useful to include this paper in the discussion.”
Response: We agree, and have added this reference, along with other recent descriptions of folliculin variants in the text on page 3, 2nd paragraph, page 5, last paragraph, and table 2; References 13-18.
Reviewer 3:

"Major comments
This study described the results of mutation analysis of the BHD gene in patients with severe COPD. The methods the authors employed, the interpretation of the results they obtained, and the conclusion they made, look sophisticated and correct. However, the basic concept and the hypothesis which made the authors initiate to examine BHD mutations in COPD patients do not seem to make sense to me. A rationale with biological or scientific plausibility must exist between a target gene (the BHD gene) and a target disease (COPD) when mutation analysis of a single gene is performed in a disease."

Response: We respectfully disagree with the reviewer and feel that there is a clear rationale for testing folliculin as a candidate gene for COPD. Numerous studies on candidate genes have been performed in COPD, and very few of them have mutations that are known to cause pulmonary disease in humans. In contrast, nearly all FLCN mutation carriers have cystic lung changes. Furthermore, it is well known that identical genetic mutations - let alone, different mutations in the same gene - may lead to different phenotypes (phenotypic heterogeneity). We further note that, while the pulmonary manifestations of FLCN mutations in the reported case series have been cysts and not emphysema, underlying emphysematous changes have been reported on biopsy specimens of non-smoking FLCN mutation carriers[1]. Finally, we note that several previous authors have hypothesized a role for FLCN in emphysema unrelated to BHD or FSP.

"Pulmonary manifestation of BHD mutation is the formation of cysts and not the development of emphysema. Radiologic appearance of cyst is totally different from that of emphysema and most of chest physicians and chest radiologists must be able to distinguish them easily. Accordingly, I can not understand the authors’ statements in conclusion (page 3) that FSP and COPD have distinct genetic causes, despite some overlap in radiographic characteristic.”

Response: We agree that the radiologic appearance of cysts is different than that of emphysema. We again note (see above), however, that underlying emphysematous changes have been reported in lung biopsies[cu3]. We also agree that chest physicians and radiologists should be able to distinguish cysts and emphysema. However, there are characteristics of cystic lung disease that do overlap with emphysema[2].

We have modified our text on page 4 to emphasize 1) our rationale for selecting this gene, and 2) the distinction between cysts and emphysema as follows: "While these cystic changes are radiographically distinct from common forms of emphysema, increasing severity of folliculin-associated cystic changes are correlated with cigarette smoking[21]. In addition, emphysema has been reported in lung resection specimens from non-smokers with folliculin mutations and FSP[7] . Some have speculated that folliculin - perhaps by regulating processes of lung growth, or altering inflammation or matrix degradation and remodelling - may also be involved in the pathogenesis of generalized, more common forms of COPD[8][7]."

"Recent studies revealed that BHD patients do not always have three phenotypic manifestations. In this context, FSP patients in whom BHD mutations were identified are
no longer considered to be FSP, instead should be diagnosed as having BHD syndrome whose phenotypic expression was limited to the lung. The authors should understand this point and change description throughout the text.”

Response: While we believe that there is still controversy on this point[3], we agree that cases of FSP may represent undetected cases of BHD. We have modified the text on page 3 to reflect this possibility as follows: "Rare, loss-of-function mutations in the folliculin gene have been found in both BHD and FSP without other BHD manifestations, suggesting a shared molecular etiology; the latter cases may also represent undetected cases of BHD[5]"

"If the authors think of possible association of BHD mutations with early-onset severe COPD, the authors should collect the phenotypic data of Boston Early-Onset COPD (EOCOPD) patients. If the authors find that a certain fraction of patients in this cohort has fibrofolliculomas or renal tumors, then BHD mutation analysis will make sense in this cohort."  
Response: While additional phenotypic data, such as screening dermatologic examinations and abdominal CT scans, would be useful, such information is not available, and since many of the early-onset COPD cases are now deceased—it would not be practical to obtain this information. As mentioned above, pulmonary manifestations are seen in most FLNC mutation carriers, while many do not have renal or dermatologic manifestations. We have provided further justification for our selection of FLNC as a COPD candidate gene above.

"In addition, the authors’ group has already published the results of genome wide linkage analysis of this cohort several times and reported that chromosome 2q and 12p showed the strong association. 
Taken together, this study lacks biological and scientific significance to analyze BHD mutation in EOCOPD patients"
Response: Many significant genetic associations have been identified in genomic regions that did not demonstrate significant linkage, and rare variants in a candidate gene of interest will not be detected with either linkage or association approaches. The presence of linkage in a region does not preclude the existence of other susceptibility variants elsewhere in the genome[4, 5]; this point has been demonstrated numerous times by recent genome-wide association studies[6], where most findings have been in areas without evidence of linkage; this point is true even for variants of strong effect, if they are rare[5], as we have previously demonstrated[7].

"Minor comments.  
1. What is the criteria to select 41 patients among EOCOPD patients for the entire BGHD gene analysis? This must be clarified”.  
Response: Our subjects were selected based on availability of larger quantities of DNA to perform DNA sequencing, as mentioned in the online supplement, second paragraph.

"2. Why 4 genotypes were selected to screen in NETT subjects and NAS subjects and to perform case-control analysis? This must be clarified. If authors would like to find possible association between SNPs in and around the BHD gene and
early-onset COPD, they should have genotypes all SNPs they found in NETT subjects and NAS subjects. The authors group has genotyped all SNPs of SERPIN2 gene and demonstrated their association with COPD. I strongly feel that this genotyping and association study is redundant."

Response: We have clarified the selection of these variants on page 6 as follows (emphasis added) "Our resequencing results suggest that COPD is not caused by rare, severely deleterious folliculin variation. However, several more common variants were identified that mapped to the folliculin transcript, raising the possibility that folliculin variants with more subtle functional impact may contribute to COPD pathogenesis. To assess this possibility, we genotyped four variants that mapped to the folliculin transcript: the two nonsynonymous variants (rs3744424 and rs41419545) and two common 5' UTR variants (rs1708629 and rs1736209)."

Genotyping a larger number (thought not all, due to linkage disequilibrium) SNPs in and around FLCN would have been necessary to rule out any evidence for genetic association. We cannot exclude the possibility that untested variants of modest effect may influence COPD susceptibility, and we acknowledge that this is a limitation on page 9, paragraph 2.

We do not feel that this study and our study of SERPINE2 are redundant; please see our comments above regarding linkage analysis.

"3. In the legend for figure 1, there is a duplication of “indicates” on the last but one line.”

Response: We have corrected this error.
