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MUC5B Promoter Variant rs35705950 Affects MUC5B Expression in the Distal Airways in Idiopathic Pulmonary Fibrosis

To the Editor:

We previously reported that the variant allele of a common *MUC5B* promoter variant, rs35705950, is significantly associated with both familial and sporadic idiopathic pulmonary fibrosis (IPF) and with increased MUC5B expression in lung tissue of unaffected subjects (1). This finding has been validated in eight subsequent independent cohorts, including our genome-wide association study (odds ratio for T [minor] allele, 4.51; 95% confidence interval, 3.91–5.21; $P = 7.21 \times 10^{-95}$) (2). Although we have found that the *MUC5B* promoter variant is associated with enhanced MUC5B expression in unaffected subjects (1) and in patients with IPF (Helling and Schwartz, unpublished results), IPF (independent of the *MUC5B* variant) is associated with enhanced MUC5B expression (1), and MUC5B message and protein are specifically expressed in the characteristic IPF honeycomb cysts (1, 3) and in terminal airway epithelia of IPF (3). However, the effect of the promoter variant on MUC5B expression has not been studied. Thus, we investigated the effect of the *MUC5B* promoter variant rs35705950 on *MUC5B* promoter activity and the distribution of

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MUC5B protein in the lung of patients with IPF. Some of the results of these studies have been previously reported in the form of an abstract (4).

Methods

Study population, lung tissue, and isolation of DNA. We obtained lung tissue from non-Hispanic white patients with IPF from the Lung Tissue Research Consortium. All participants provided informed consent. Six homozygous (GG, major allele), six heterozygous (GT), and six homozygous (TT, minor allele) samples for the variant rs35705950 were selected to be frequency matched for age, sex, and smoking status.

Cloning and transfection. A 4.2-kb region 5′ to the transcription start site of the *MUC5B* gene that includes the rs35705950 variant was amplified using the Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). The polymerase chain reaction amplicons were cloned into pCR2.1-TOPO vector (Life Technologies, Carlsbad, CA), and site-directed mutagenesis was performed to introduce an Nhe-I restriction site at the ATG site for subcloning. Cloned sequences were confirmed by Sanger sequencing, and the *MUC5B* promoter area was subcloned into the pGL4.10 vector (Promega, Fitchburg, WI). Site-directed mutagenesis was also performed to convert the rs35705950 variant (GG to mutated-TT and TT to mutated-GG). All plasmids used for transfection experiments were prepared using an endofree plasmid maxi kit (Qiagen, Dusseldorf, Germany). Each plasmid was assayed six times in three separate experiments, using standard transfection techniques and A549 cells.

Immunohistochemistry. Standard staining approaches were used on lung tissue sections from the same patients used for the promoter assays. Quantitative analysis was performed by three independent, blinded observers with a point-counting method using the STEPanizer software (5).

Results

We identified 16 variants in the 4.2-kb 5′ promoter region of *MUC5B* within the six GG and six TT study subjects with IPF. Each subject had a unique haplotype for the putative *MUC5B* promoter region. Fifteen variants were previously known, and one variant was novel. Subsequent to cloning, homozygous plasmids were used for the remaining experiments.

To determine whether the *MUC5B* promoter variant modulates *MUC5B* promoter activity, we performed a standard luciferase assay with the plasmids from 12 subjects (GG [n = 6] and TT [n = 6]) with IPF. We excluded one sample as an outlier because the sample repeatedly showed much higher luciferase activity than all other samples. Results demonstrated that the *MUC5B* promoter activity of the TT group was significantly higher than that of the GG group (nonparametric $P = 0.002$; Figure 1).

To account for the potential effect of other promoter variants and to examine the sole effect of rs35705950, we performed site-directed mutagenesis and created plasmids that had the opposite genotype for rs35705950. When the wild type was converted to the rs35705950 variant (G to T), *MUC5B* promoter activity was significantly increased (nonparametric $P < 0.05$). In contrast, when the rs35705950 variant was converted to the wild type (T to G), *MUC5B* promoter activity was significantly decreased (nonparametric $P < 0.01$), again demonstrating the requirement of the T allele for enhanced promoter function.

To test whether the *MUC5B* promoter variant rs35705950 alters the distribution of MUC5B in IPF lung, we performed quantitative histopathological analysis of IPF lung tissue using surgical samples obtained from the same subjects included in the promoter assays. We found that the percentage of fibrosis correlated positively and significantly with honeycomb cyst lumen and correlated negatively and significantly with alveolar areas. In addition, there were no differences in morphometric counts for each lung structure between the GG and TT groups, including the epithelium of cystic structures (honeycomb cysts and alveolar cysts). These findings confirmed the presence of IPF and

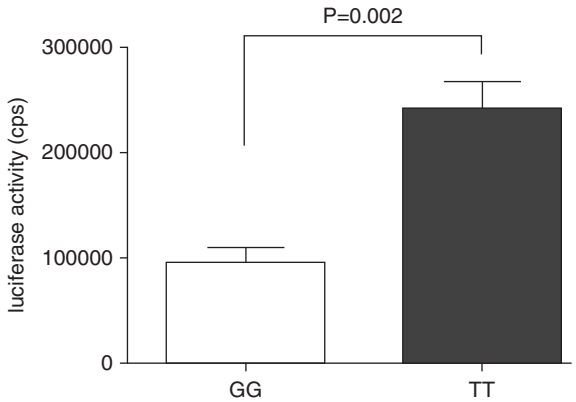

Figure 1. Effects of site-directed mutagenesis of the *MUC5B* promoter variant. *MUC5B* promoter activity of the TT group was significantly higher than that of the GG group in the A549 cell line. Data are represented as mean ± SEM (n = 5, 6) and significance assessed with an unpaired one-tailed Mann-Whitney *U* test ($P = 0.002$).

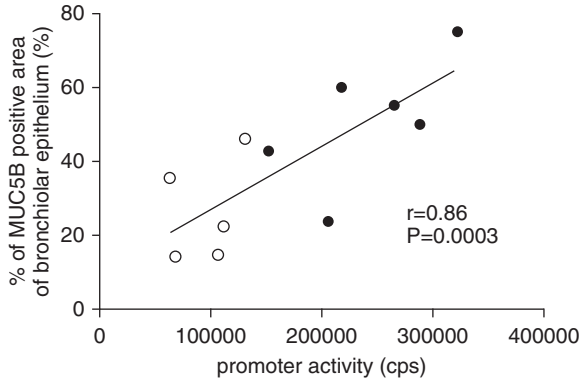

Figure 2. Correlation between *MUC5B* promoter activity and percentage of MUC5B-positive area of the small airway epithelium in idiopathic pulmonary fibrosis lung. *Solid circles* represent GG samples, and *open circles* represent TT samples. A Spearman correlation coefficient test (one-tailed) was used to analyze these data. $r = 0.86$; $P = 0.0003$.

that the interrogated tissues demonstrated similar disease involvement.

In contrast, the percentage of MUC5B-positive area in the epithelial cells of structurally intact bronchioles from the TT samples was significantly higher than that of GG samples (nonparametric $P < 0.05$). Furthermore, the percentage of MUC5B-positive area in the epithelial cells of the bronchiole was strongly correlated with the *MUC5B* promoter activity ($r = 0.86$; nonparametric $P = 0.0003$; Figure 2). To further understand the influence of rs35705950 on MUC5B expression in the peripheral airway, we examined the distribution of MUC5B in GT heterozygotes and found that, similar to subjects with the TT genotypes, the GT heterozygotes have significantly more MUC5B-positive staining in the epithelial cells of the bronchioles when compared with lung samples from GG subjects (nonparametric $P < 0.005$). Collectively, these findings indicate that the presence of the *MUC5B* promoter variant rs35705950 contributed to specific MUC5B protein expression in epithelial cells within the bronchioles in the IPF lung.

Discussion

Our findings demonstrate that the presence of the *MUC5B* promoter variant rs35705950 increases the activity of the *MUC5B* promoter and contributes to the expression of MUC5B in the IPF lung, particularly in the bronchiolar epithelium. Although there have been many studies establishing the association of the *MUC5B* promoter variant with IPF, this is the first study to demonstrate the contribution of rs35705950 to both *MUC5B* promoter activity and the distribution of MUC5B protein in the IPF lung. These findings further support the association of rs35705950 and overexpression of MUC5B with IPF, but future studies are needed to establish support for a mechanistic and potentially pathogenic role. ■

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Neurocritical Care Society Views on "Potentially Inappropriate Treatments in Intensive Care Units"

To the Editor:

On behalf of the Neurocritical Care Society (NCS), we wish to offer our perspective and commentary on the ATS/AACN/ACCP/ESICM/SCCM policy statement on how to handle requests for potentially inappropriate treatments in intensive care units, published in the June 1, 2015, issue of the *Journal* (1). This position paper affirms the importance of proactive communication and expert involvement in the prevention of disputes of intractable treatment conflicts, as well as the development of a fair, transparent, multidisciplinary process of dispute resolution regarding potentially inappropriate therapy with options for transfer, the input of a second medical opinion, and the right for extramural appeal.

Perhaps the most significant achievement of this work, and also that which is most likely to garner dissent, is that after decades of scholarly discourse on medical futility, there is a consensus on restricting the definition of "medical futility" to a physiologic sense of the term. This policy statement creates two distinct categories: interventions that cannot achieve their intended biologic goal (futile interventions), and interventions whose application is inherently value laden, and thus a provider's refusal to perform them, despite the request of a patient, ought to require a resolution process by a third party (potentially inappropriate treatments).

To explore how the members of the NCS would respond to such a position, we conducted an open survey of our members. Rather than directly asking them which types of therapy need not be offered or refused, we developed a set of eight common clinical vignettes (*see* online supplement) with the care pathway chosen by the patient or surrogate. Half were oriented to physiologic futility and half to value-laden questions. Survey respondents chose from a six-item scale ranging from "refuse" to "highly recommend." Our focus was to examine whether members had a greater consensus (similarity in response patterns) regarding the physiologic questions versus the quality-of-life questions. There was a statistically significant difference in the distribution of responses to the questions analyzed, using Kruskal-Wallis test for nonparametric data ($n = 44$; $P < 0.0001$), indicating a greater consensus on questions regarding physiologic futility.

This letter has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org