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Surfactant protein B intron 4 variation in German patients with COPD and acute respiratory failure

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Abstract. Chronic obstructive pulmonary disease (COPD) is a major health problem. Genetic factors that contribute to the disease have been postulated. The pulmonary surfactant protein B (SP-B), which is essential for normal lung function, is considered as a candidate gene for COPD in this case-control study. We studied the SP-B intron 4 size variants in 346 individuals. This group consisted of 118 patients with chronic bronchitis or COPD, including 24 patients with acute respiratory failure (ARF) in COPD, 118 matched controls without pulmonary disease and 110 healthy individuals (population control). The frequency of intron 4 variants was similar in either control group (10.9%, 14.4%, respectively), with a small increase in the COPD group (18.6%). This increase was due to a high increase of intron 4 variants in the ARF subgroup (37.5%, $p = 0.003$, OR 4.9, 95% CI: 1.76–13.6). The data indicate that SP-B intron 4 variants may associate with increased risk of ARF in COPD and may be used as a marker of susceptibility in this disease subgroup.

Keywords: Chronic bronchitis, SP-B, polymorphism, acute respiratory failure

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major health problem. It causes severe morbidity and mortality in all industrial countries and is one of the leading causes of death [3]. COPD with multifactorial and/or multigenic etiology is characterized by the presence of chronic airway obstruction due to chronic bronchitis or emphysema. Risk factors for COPD include cigarette smoking, occupational and environmental exposure to dust and gases, as well as a genetic predisposition [28]. Cigarette smoking is strongly associated with the development of COPD and can induce alterations of the surfactant system [7,22]. In the airways of patients with COPD, local conditions are changed that impair local host defense and facilitate infections. One of the factors promoting bacterial adherence is impaired mucociliary clearance [17]. The bronchiolar surfactant layer reduces sputum adherence and has been demonstrated to increase mucociliary clearance [1,31] and stabilize airways [23].

A decrease in the amount of bronchial surfactant has been demonstrated by several investigators [15] in patients with chronic bronchitis. Surfactant treatment improves both sputum mucociliary transportability and lung function in these patients [2].

Pulmonary surfactant is a complex mixture of lipids, primarily phospholipids and surfactant proteins (SP)-A, B, C and D. The structural and functional integrity of

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surfactant depends on the surfactant proteins A, B, and C. Surfactant is essential for normal lung function and components of surfactant play a role in host defense and inflammatory processes of the lung. Although the main function of surfactant is to lower the surface tension at the air/liquid interface, and thereby prevent alveoli from collapsing [10], a number of other functions have been attributed to surfactant. A bronchiolar surfactant layer has been demonstrated [24,36], that appears to be important for several physiological functions, such as interaction with inhaled particles and improvement of bronchiolar clearance of the upper respiratory tract [1,31]. Thus, it is possible that surfactant dysfunction may be a contributory factor to the development of chronic airway obstruction and/or chronic bronchitis. Therefore, because of the importance of surfactant in normal lung function and alveolar integrity the surfactant protein genes may be good candidates in the study of markers for COPD.

Surfactant protein B is important for the formation of the active surfactant surface film [32] and for normal lung function. Neonatal rabbits treated with monoclonal antibody to SP-B exhibit reduced compliance, inflammation, and hyaline membranes [29], as well as reduced tidal volumes. SP-B $(-/-)$ knockout mice die from severe respiratory failure [5], and the heterozygous SP-B $(+/-)$ mice exhibit decreased lung compliance and increased residual lung volume [4].

The gene for the SP-B has been localized on the short arm of chromosome 2 [6,34]. It consists of 11 exons with the first 10 encoding the precursor protein [27]. Different polymorphisms within the SP-B gene have been described [11,20,21]. SP-B or SP-B-linked gene variants have been associated with several diseases, such as respiratory distress syndrome (RDS) [8,9,11] congenital alveolar proteinosis [20,25], and COPD in Mexicans [13].

Because of the critical importance of SP-B in surfactant function we hypothesized that SP-B polymorphisms can identify pulmonary disease subgroups. To investigate this hypothesis we studied SP-B gene variants within the intron 4 in patients with chronic bronchitis, including acute respiratory failure in COPD. Subsequently, we determined the frequency of these variants in subgroups of patients with pulmonary disease, in healthy persons who were age, sex, and smoking status matched to the patients, and in randomly picked control subjects.

The data revealed an increased frequency of intron 4 variants in patients with COPD, due to a high increase of these variants in patients with acute respiratory failure in COPD, suggesting an association between this subgroup of patients and the SP-B locus and/or its flanking loci.

2. Patients and methods

2.1. Patients

In the present case-control study, we analyzed 346 Caucasian subjects. These subjects were divided into the patient-group (COPD), which in turn was divided in different subgroups based on severity, and two control-groups (population and control-group). The characteristics of the groups studied are summarized in Tables 1 and 2, and described below. The age, sex, and smoking status of patients were matched to that of controls and healthy individuals. Smoking habits were classified in two categories, those who never smoked or smoked fewer than 5 pack years (non-smokers) and persons with a history of smoking over 5 pack years and persistent smokers (smokers). Blood specimens were obtained for genotype analysis with permission of the patients and according to the institutional board review.

2.1.1. Population group

This group consisted of 110 randomly selected healthy persons. These had a health checkup upon entering a new work position or changing work positions between October and December 1998 at the University of Marburg. The age of the 110 patients, 55 males and 55 females, ranged between 20 and 62 years, with a mean age of 32.2. Of these individuals 43 (39%) were persistent smokers or had a history of smoking over 5 pack years.

2.1.2. Control group

This group served as the "matched" control to the cases. It consisted of 118 subjects from the department of internal medicine, without any history or symptoms of respiratory disease. We included patients between January 1998 and January 1999. The age, sex, and smoking status of these subjects were matched to those of the patients. For all these control patients a measurement of airway function was performed by spirometry and whole-body plethysmography. Only patients with normal lung function were included. One hundred and one patients (85.5%) suffered from coronary heart disease without heart failure, 5 patients (4.2%) from sleep apnea, 7 patients (5.9%) underwent surgery for cancer

Table 1
Group characteristics of the patient and control groups

Group/ characteristic	Population	Control	Chronic bronchitis	COPD I–III°	Acute respiratory failure
Number	110	118	118	94	24
Male:Female	1.0 (55/55)*	3.9 (94/24)	3.9 (94/24)	4.2 (76/18)	3.0 (18/6)
Age (Range)	32.2 (20–62)	68 (35–87)	68 (35–87)	67.6 (35–87)	69.7 (55–84)

*Ratio of male to female (M/F).

Table 2
Smoking status* of subjects in the different groups

Smoking habits	Population	Control	Chronic bronchitis	COPD I–III°	Acute respiratory failure
Smokers	39% (43)	77.9% (92)	77.9% (92)	77.9% (73)	79.1% (19)
Non smokers	60.9% (67)	16.1% (19)	16.1% (19)	15.9% (15)	16.6% (4)
Unknown	0% (0)	5.9% (7)	5.9% (7)	6.3% (6)	4.1% (1)

*Smokers are classified by a minimum of 5 Pack Years.
**In each category the number of subjects is shown in parenthesis.

and 5 patients (4.2%) suffered from other diseases such as diabetes mellitus. Similar to the chronic bronchitis group, 94 males and 24 females, with a mean age of 68 years, ranging from 35–87 years, were included. Of these patients 92 (77.9%) were persistent smokers or had a history of smoking for more than 10 pack years.

2.1.3. Chronic bronchitis group

The chronic bronchitis group, consisted of 118 patients, 94 males and 24 females, with the classical history of chronic bronchitis according to the definition by the world health organization: Cough and expectoration presented on most days for a minimum of three months in two consecutive years and could not be attributed to other pulmonary or cardiac cause [3].

The patients had histories of chronic or recurrent cough and expectoration and/or dyspnea on effort, wheezing or breathlessness. All of the patients received a physical examination, and underwent measurement of airway obstruction by spirometry and whole-body plethysmography. According to the international diagnostic criteria [3], we used the following criteria for diagnosis and used the best of three acceptable maneuvers: Forced expiratory volume in 1 second (FEV_1), FEV_1 to forced vital capacity (FEV_1/FVC) and peak expiratory flow (PEF): $FEV_1 < 70\%$, $FEV_1/FVC < 80\%$, peak flow $< 70\%$. Patients presenting a minimum of two diagnostic criteria and history of chronic bronchitis were included in the chronic bronchitis group. These patients were subclassified by severity, using FEV1, according to the international classification. From the 118 patients, 46 (38.9%) belonged to COPD group I ($FEV_1 > 50\%$), 33 (27.9%) to COPD group II (FEV_1 35–49%), and 15 (12.7%) to COPD group III ($FEV_1 < 35\%$). The subgroup of highest severity consisted of 24 (20.3%) patients with acute respiratory failure in COPD and is described below.

Smoking or a history of smoking (minimum of 5 pack years) was described for 92 subjects (77.9%), and a non-smoking history for 19 (16.1%). Only patients with a classical history of chronic bronchitis were included. Patients with a history of allergic asthma or atopy, or any active pulmonary disease, such as lung cancer, pneumonia, tuberculosis, or cystic fibrosis were not included.

2.1.4. COPD subgroup: Acute respiratory failure

This subgroup of the chronic bronchitis group consisted of 24 patients with acute respiratory failure in COPD. All these patients were diagnosed previously with COPD by a long, typical history of the disease and measurement of airway parameters. These patients had acute respiratory failure due to acute exacerbation of their chronic airway disease and needed to be treated at the ICU. Patients being treated from January to September 1998 at the intensive care unit of the division of respiratory medicine at the university of Marburg were included. The group comprises only patients with acute respiratory failure and requirement of respirator ventilation at ICU. This group consisted of 18 males and 6 females, with the mean age of 69.7 years. In this subgroup smoking or a history of smoking was determined for 19 (79.1%) patients.

2.2. PCR and analysis

DNA was prepared from blood specimens using a kit provided by Qiagen (Hilden, Germany).

We used specific PCR to genotype intron 4 variation. For intron 4 specific PCR primers 161 (5'-TGTGTGTGAGAGTGAGGGTGTAAG-3'; antisense, position 2135-2158) and 172 (5'-CTGGTCATC GACTACTTCCA-3'; sense position 1552–1571) were used in a reaction volume of 50 μl. The nucleotide positions are according to Pilot-Matias et al. [27]. PCR products were generated with 10 ng DNA as template, 0.2 units Taq polymerase provided by Boehringer Mannheim (Mannheim, Germany), 1.5 μl buffer 1 (17.5 mM $MgCl_2$) and buffer 2 (22.5 mM $MgCl_2$) 10 $\times$ concentrated, provided by Boehringer Mannheim (Mannheim, Germany), 1 μl of each specific primer (100 ng/μl) 161 and 172, 1 μl dNTPs (1.25 mM each dNTP).

The samples were heated without Taq polymerase for 10 minutes to 95°C, then Taq polymerase was added, followed by 30 cycles of 94°C 30 sec, 59°C 1 min, 72°C 1 min, and a final cycle of 5 min 72°C. As size marker we used cloned and sequenced intron 4 fragments as described previously [11,35]. The PCR-products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

2.3. Statistics

The statistical analysis was performed by means of the SPSS software package (Version 9.0, SPSS Inc., Chicago, IL, USA). The frequency of the SP-B variants in the respective groups (control, population, chronic bronchitis, ARF) was compared using the Chi-squared test analysis. Differences in the frequency of variant vs. invariant were considered statistically significant when p was $\leqslant 0.05$ in the Chi squared Test or Fisher's Exact test when appropriate. The 95% confidence interval (95%CI) for the given odds ratio is shown.

3. Results

3.1. Description of intron 4 variants in the groups under study

The intron 4 SP-B gene variation is characterized by deletions or insertions, as shown schematically in Fig. 1. The size of intron 4 is about 600 bp, and this represents the size of the invariant allele. Approximately, the first half of intron 4 consists of 11 motifs [11]. Each motif consists of a 20 bp conserved element followed by a variable number of CA-repeats. Variants are derived by the gain or loss of certain motifs. The number of deleted or inserted motifs ranges from -8 to $+7$ [11,35]. Figure 1, Panel A depicts the schematic representation of these polymorphisms and Panel B depicts the invariant band with a size of about 600 bp (arrow) and the variant band with either a smaller (lanes 2–3) or a larger (lanes 5–8) size. We used cloned and sequenced DNA as size markers to define the common deletions as type I or II, lacking 5 or 8 motifs, respectively (Panel A). In the present study we pulled all the deletion variants into one group and all the insertion variants into another.

3.2. The frequency of intron 4 variants differs among various groups

The frequency of intron 4 variants was analyzed in 346 individuals divided in two control and one disease group with different subgroups (see Methods). The frequency of SP-B variants (deletion or insertion) in the two control groups was similar (10.9% and 14.4%) (Table 3). A small increase (18.6%) was observed in the chronic bronchitis group and no difference was detected in COPD subgroups I–III (13.8%) compared to controls. A small decrease in the frequency of the variant was observed in group III° (I°: 15.2%, II°: 15.1%, III°: 6.6%) compared to either control (10.9%, 14.4%). However, the frequency of the variant SP-B alleles was significantly higher in the acute respiratory failure group (ARF) compared to either control group ($p = 0.003$ for population and $p = 0.017$ for control). The variant alleles were present in 37.5% of the ARF-patients compared to 14.4% and 10.9%, in the matched control patients and the population group, respectively (Tables 3 and 4, Fig. 2). The odds ratio for the comparison of the ARF-group with the population control was 4.9 (95% CI $= 1.83 - 13.6$) and with the matched control was 3.55 (95% CI $= 1.4 - 9.4$) (Table 4). No significant differences were observed between COPD (groups I°–III°) and either control group (data not shown).

Subdividing by gender revealed differences in the frequency of SP-B gene variants. In ARF patients the percent of SP-B variants was increased in females (50%) compared to males (33.3%) when compared to either control group (Table 3). All of the samples with the variant allele were heterozygous, carrying one variant and one invariant allele, except for one sample that was homozygous for the smaller size variant (deletion), with both of the alleles being Type I alleles. Among the 49 patients with variant alleles, 30.6% were due to insertions and 69.3% were due to deletions. There was

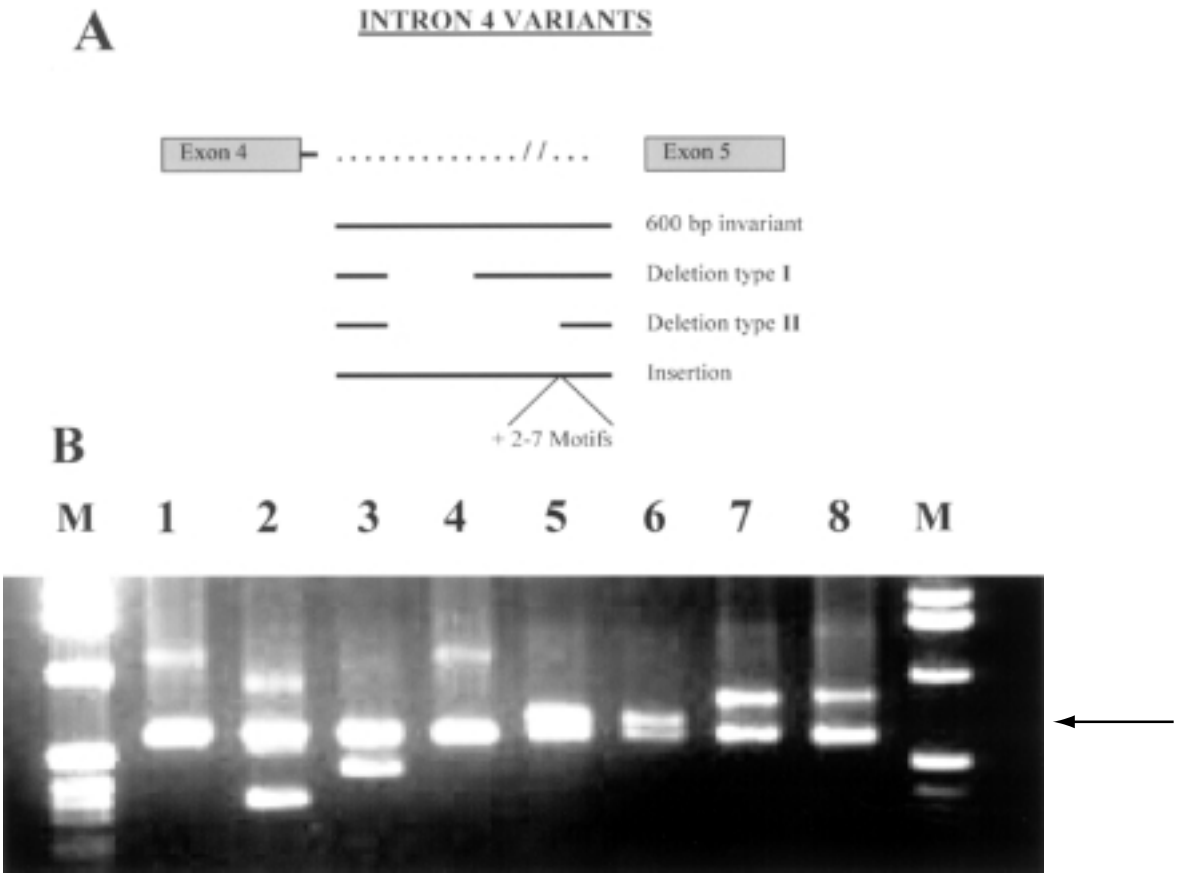

Fig. 1. Intron 4 group variants. (A) Depicts schematically length variants. Intron 4 or the invariant segment is approximately 600 bp. The first half of intron 4 consists of motifs as described in Results. The two common deletion variants that lack 5 or 8 motifs are described here as type I and type II, respectively. The larger size variants are characterized by insertion of several motifs. (B) Depicts intron 4 PCR products by agarose gel electrophoresis. M = molecular weight markers. Numbered lanes depict one invariant band (arrow). Lanes 2 and 3 show type II and type I deletions respectively (lower bands). Lanes 5–8 depict variants with different insertions (5 = +2 motifs, 6 = +3 motifs, 7 = +6 motifs, 8 = +7 motifs).

Table 3
Percent of variants in patient and control groups

Variation	Population	Control	Chronic bronchitis	COPD I–III[o]	Acute respiratory failure
All variants	10.9% (12)*	14.4% (11)	18.6% (22)	13.8% (13)	37.5%** (9)
Deletion	66.6% (8)	81.8% (9)	63.6% (14)	61.5% (8)	66.6% (6)
Insertion	33.3% (4)	18.8% (2)	36.3% (8)	38.4% (5)	33.3% (3)
Male	12.7%	15.9%	19.1%	15.7%	33.3%
Female	9.0%	8.3%	16.6%	5.5%	50%

In the first 3 rows the number of subjects is shown in parentheses.
$^{**}p = 0.003/p = 0.017$ (population / control versus ARF).

no significant difference in the frequency of alleles with insertions or with deletions among the different groups. From the 31 alleles with deletions, 23 corresponded to type I, and 8 to type II deletions. These data together suggest that the SP-B intron 4 variants may serve as a marker for acute respiratory failure in COPD.

4. Discussion

In the present study we investigated the frequency of SP-B intron 4 variants in various groups with pulmonary disease. We observed a significant difference in the frequency of the SP-B intron variants between

Table 4
ARF versus control and population groups

	Control %(n)	ARF %(n)	Odds ratio	95% CI of odds ratio	p-value	Fisher's exact test
Insertion/deletion	14.4% (17)	37.5% (9)	3.55	1.4–9.4	$p = 0.008$	$p = 0.017$
	Population %(n)	ARF %(n)	Odds ratio	95% CI of odds ratio	p-value	Fisher's exact test
Insertion/deletion	10.9% (12)	37.5% (9)	4.9	1.8–13.6	$p = 0.001$	$p = 0.003$

*In the Control and ARF columns the number (n) of individuals carrying the SP-B variant is shown in parenthesis.

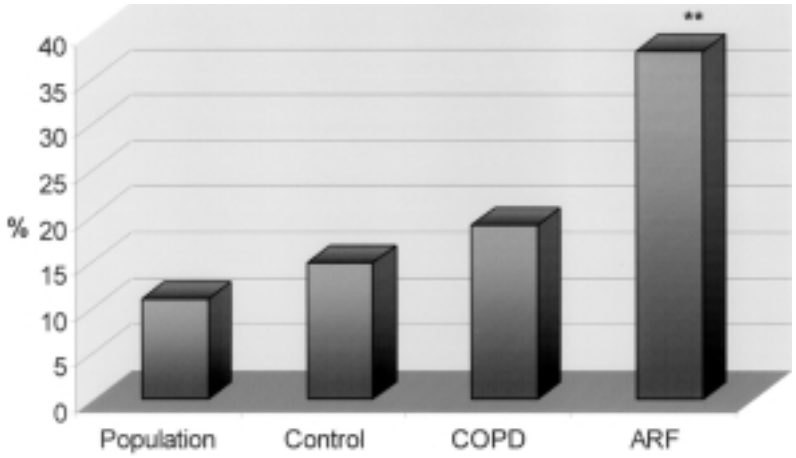

Fig. 2. Percent of the variant intron 4 fragment in different groups. The percent (%) of the variant fragment in the different groups under study is shown graphically. $^*p = 0.003/0.017$ (population/control versus ARF, Fisher's Exact Test).

healthy persons and patients with acute respiratory failure (ARF) in COPD. The increased frequency of these variants was even higher in a subgroup of females with ARF in COPD compared to males.

We focused our attention on SP-B, because SP-B is necessary for the formation of the active surface film and essential for life [5]. Also, associations of SP-B markers within the coding and flanking regions have been observed with COPD in a Mexican study group [13] when compared to a Mexican smoker control group. The Mexican study group stratification differed in part from the present study and the intron 4 variants were not investigated in the Mexican study. However, our unpublished findings (Floros) indicate that there was no difference in intron 4 variants between COPD and controls in the Mexican study, and also that there may be a lower frequency of intron 4 variants in Mexicans. It is unknown whether this observation reflects differences in the study group stratification between the two studies or ethnic differences. With regard to the latter possibility, it is of interest to note that ethnic or race differences in the frequency of intron 4 have been observed previously [8,11,14]. The frequency of intron 4 variants appears to be lower in a Finnish study group compared to white American study groups. Also differences in the frequency and type of SP-B intron variants in blacks and whites with RDS have been observed.

The size variability within intron 4 is caused by the gain or loss of motifs [11], consisting of a 20 bp conserved sequence followed by various dinucleotide repeats (CA-repeats). Di-, tri-, and tetranucleotide repeats have been shown, in some cases, to be associated with neurodegenerative diseases, such as Huntington chorea and fragile-X-syndrome [16]. The SP-B intron 4 variability is meiotically stable and an association with respiratory distress syndrome has been demonstrated [8,11]. Currently, it is not known whether the intron 4 variability has any impact on SP-B gene expression or regulation. It is also unknown whether the SP-B locus itself or a linked gene contributes to COPD and/or to severe cases of COPD. However, because this variability results in large deletions or insertions, it is possible that these alterations compromise splicing and/or SP-B mRNA processing that may result in altered SP-B mRNA and/or lower amounts of SP-B pro-

tein. Recently, an SP-B mRNA splice variation with disease implication for pulmonary diseases has been shown [21] and abnormality in a region that spans this splice variation has been implicated in congenital alveolar proteinosis [19]. It is possible that SP-B intron 4 variants may have an impact on the amount of SP-B protein. This in turn may alter the relative component composition of surfactant and/or structure of functional surfactant.

The consequences of a disturbed or deficient bronchiolar surfactant layer may lead to mucus accumulation and a deficiency in the first line of host defense in the upper respiratory tract, as well as enhanced bacterial adherence. These are major factors in the pathophysiology of chronic bronchitis, and may contribute to the development of acute respiratory failure in COPD. Of interest, although SP-B is synthesized by alveolar type II cells and bronchiolar epithelial cells [26], recent studies indicate that the function of alveolar and bronchiolar SP-B may differ [18]. The role of bronchiolar SP-B is currently unknown, but bronchiolar SP-B cannot substitute for the function of alveolar SP-B. Moreover, reduced levels of SP-B have been observed in heterozygous SP-B knockout mice ($-/+$), which show small lung physiological abnormalities [4], and enhanced susceptibility to oxidant lung injury [33]. These observations may have relevance to chronic bronchitis where a decrease in the amount of bronchial surfactant has been observed [15] and where surfactant therapy appears to improve sputum mucociliary transportability and lung function [2].

Case-control studies may identify "spurious" associations due to population admixture [30]. We attempted to control for this by studying subjects with the same ethnic background and by using two control groups, a population control and a patient matched control group. However, although the findings in this study are intriguing, given the small sample size of the acute respiratory failure group, these findings should be confirmed by additional studies with a larger sample size and strict epidemiologic criteria.

It is possible that the marker polymorphism studied here serves as a marker of respiratory problems and may not be specific to COPD. In support of this possibility are the findings where the frequency of the intron 4 variant is increased in white and black subjects with neonatal respiratory distress syndrome (RDS) of a certain gestational age and/or sex [8]. Therefore, it is most likely that SP-B contributes to changes in the local condition of airways that in turn may lead to pulmonary disease and to COPD, in particular, only under certain conditions that may include interaction with alleles of other genes in a time point dependent manner [8,12]. This time point may reflect a point during development or a point in the progression of the disease. Moreover, given the available information, these disease-associated time-dependent allele interactions may be further modulated by ethnic, race, or sex differences.

In summary, following investigation of differences in the frequency of the SP-B intron 4 variants between control and disease groups, we observed that the frequency of this variant is increased in a subgroup of COPD, the acute respiratory failure. We speculate that the data presented here along with the published information point to the possibility that association of this variant with a given pulmonary disease subgroup is influenced by ethnic and race dependent factors as well as developmental time points and/or time points along the progression of a given disease. Further studies are also necessary to determine the impact of this polymorphism on protein expression and disease pathogenesis, and/or determine how this polymorphism could mechanistically be linked to acute respiratory failure in COPD.

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