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Allele-specific N-glycosylation delays human surfactant protein B secretion *in vitro* and associates with decreased protein levels *in vivo*

Saija Taponen[1], Johanna M. Huusko[1], Ulla E. Petäjä-Repo[2], Reija Paananen[1,3], Susan H. Guttentag[4], Mikko Hallman[1,5] and Ritva Haataja[1,6]

BACKGROUND: Surfactant protein B (SP-B) is essential for normal lung function, and decreased concentrations of SP-B have a deleterious effect on pulmonary outcome. SP-B levels may correlate with variations in the encoding gene (*SFTPB*). *SFTPB* single-nucleotide polymorphism Ile131Thr affects proSP-B N-glycosylation in humans and the glycosylated Thr variant associates with pulmonary diseases.

METHODS: We analyzed SP-B levels in amniotic fluid samples for associations with *SFTPB* polymorphisms and generated cell lines expressing either proSP-B/131Ile or proSP-B/131Thr for examining the effect of glycosylation on proSP-B secretion kinetics. To determine any transcription preference between Ile131Thr allelic variants, we used heterozygous human lungs for allelic expression imbalance assays.

RESULTS: Protein levels correlated with Ile131Thr genotype and the lowest SP-B levels were observed in Thr/Thr homozygotes. Our results suggest that Ile131Thr variation–dependent N-glycosylation associates with decreased levels of SP-B, which is secreted from fetal lung to amniotic fluid. Glycosylated proSP-B/131Thr was secreted from transfected cells at a lower rate than nonglycosylated proSP-B/131Ile. Expression levels of the mRNA variants were equal. Secretion of the glycosylated variant was thus delayed *in vitro* by a posttranscriptional mechanism.

CONCLUSION: These data support the hypothesis that proSP-B glycosylation due to Ile131Thr variation may have a causal role in genetic susceptibility to acute respiratory distress.

Pulmonary surfactant, a complex mixture of phospholipids, neutral lipids, and proteins, is produced, assembled, and secreted by type 2 alveolar epithelial cells in the alveolar epithelial lining (1,2). Its main function is to lower surface tension by forming a surface-active film, and absence or dysfunction of this film results in alveolar collapse during expiration. This is seen in premature infants with respiratory distress syndrome (RDS), which is caused mainly by a deficiency of pulmonary surfactant at the time of birth (2,3). In addition, pulmonary surfactant is a critical component of the innate immune defense system (3).

Surfactant protein B (SP-B), a highly hydrophobic minor component of pulmonary surfactant, is crucial for normal lung function (2,4–6). In addition to its surface tension–lowering properties and essential functions related to surfactant metabolism (7), SP-B has roles in host defense (8,9). Mature SP-B can be detected in developing human lungs at 24 wk of gestation, although only at 3% of adult levels. SP-B in amniotic fluid originates from the fetal lung (10).

Bioactive SP-B is a homodimer ~18 kDa in size. After signal peptide cleavage, and on its route from the endoplasmic reticulum (ER) to intracellular lamellar bodies, processing of 42-kDa proSP-B to the mature 8-kDa SP-B monomer involves several cleavage steps. The initial NH_2-terminal cleavage, which results in a 25-kDa intermediate, is independent of cell type, whereas the more distal processing stages are specific to type 2 cells and are performed by cell type–specific proteases (6,11–13). The active peptide is stored in lamellar bodies and secreted with phospholipids to the airway lumen (14).

N-glycosylation is an important co- and posttranslational modification that performs diverse cellular functions. Human proSP-B contains two sites for N-linked glycosylation: in the NH_2 terminus at Asn129 and in the COOH terminus at Asn311 (6). The fragments of proprotein containing these sites are cleaved before the formation of mature SP-B polypeptide. Glycosylation at Asn129 is specific to humans and is dependent on the nonsynonymous single-nucleotide polymorphism Ile131Thr (rs1130866) in the SP-B–encoding gene *SFTPB* (15,16).

The *SFTPB* 131Thr variant polypeptide is glycosylated, and the Thr allele is associated with several pulmonary diseases such as neonatal RDS, acute RDS in adults, and idiopathic pulmonary fibrosis in smokers (15,17–21). Given its high global frequency of 0.46 in the population according to the Short Genetic Variations

The first two authors contributed equally to this work.
[1]Department of Pediatrics, Institute of Clinical Medicine, University of Oulu, Oulu, Finland; [2]Department of Anatomy and Cell Biology, Institute of Biomedicine, University of Oulu, Oulu, Finland; [3]Child and Adolescent Health and Welfare Unit, National Institute for Health and Welfare, Oulu, Finland; [4]Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania; [5]Department of Children and Adolescents, Oulu University Hospital, Oulu, Finland; [6]Biocenter Oulu, University of Oulu, Oulu, Finland.
Correspondence: Johanna M. Huusko (johanna.huusko@oulu.fi)

Received 28 November 2012; accepted 18 April 2013; advance online publication 9 October 2013. doi:10.1038/pr.2013.151

database (dbSNP) by the National Center for Biotechnology Information (22), we hypothesized that this single-nucleotide polymorphism most likely becomes detrimental only under certain disease-predisposing conditions such as transient surfactant deficiency shortly after birth and in interaction with other potentially disease-predisposing genetic variants like those encoding surfactant protein A. Under these conditions, the allele-dependent glycosylation status at Asn129 could have an effect on proSP-B stability, processing, secretion, and/or folding and would thus result in altered mature SP-B levels among individuals carrying different genotypes (15,17). In addition to Ile131Thr (rs1130866), other potentially disease-associating *SFTPB* polymorphisms that have been assumed to have an effect on transcriptional activity or mRNA splicing include two variations in the promoter region (rs2077079 C/A at −18 and rs3024791 G/A at −384) and a length variation in intron 4 (23–27). These polymorphisms could contribute to individual differences in mRNA and protein levels *in vivo*. The concentration of mature SP-B in the epithelial lining may depend upon the *SFTPB* genotype, which could have a causal influence on lung function and pulmonary morbidity. Observations in conditional transgenic adult animals indicate that reduced SP-B protein expression and decreased SP-B concentration in bronchoalveolar lavage fluid are associated with risk for respiratory failure, suggesting that there is a critical threshold of 50% for alveolar SP-B content that is necessary for normal pulmonary function and gas exchange (28).

In this study, we explored the relative levels of SP-B in amniotic fluid collected from pregnancies at the time of delivery for evaluating whether protein levels correlate with any of the *SFTPB* polymorphisms that are considered functional. In addition, we determined whether the glycosylation status at Asn129 affects the rate of SP-B secretion, reflecting potential differences in SP-B processing or secretion kinetics between the allelic variants. Finally, we analyzed the allelic expression of the *SFTPB* mRNA in Ile131Thr heterozygous adult lung tissue samples to quantitate the relative expression of the two variants. Our results are consistent with the initial hypothesis that Ile131Thr allele–specific glycosylation of the proSP-B peptide reduces the production or delays the secretion of mature SP-B through a posttranscriptional mechanism.

RESULTS

Correlation Between Amniotic Fluid SP-B Levels and *SFTPB* Polymorphism

We used western analysis for SP-B quantitation, and the validity of the assay was explored as described in the **Supplementary Data** online. To determine whether a certain *SFTPB* polymorphism correlates with protein levels *in vivo*, the SP-B levels in amniotic fluid were plotted against the *SFTPB* genotypes.

Amniotic fluid SP-B levels differed significantly between the Ile131Thr (rs1130866) genotypes (ANOVA $P = 0.013$; Kruskal–Wallis $P = 0.016$), whereas there were no correlations between protein levels and genotypes with the other polymorphisms (**Table 1**). The Thr/Thr genotypes showed lower SP-B levels as compared with the other Ile131Thr genotypes ($P < 0.05$). The relative protein level seemed slightly higher in the heterozygous Ile131Thr group than in the homozygous Ile/Ile group, but the difference was not statistically significant ($P > 0.1$), suggesting that the relative SP-B levels were similar in the Ile/Ile and Ile/Thr genotypes. Infants with the Thr/Thr genotype tended to have higher incidence (12.5%) of respiratory distress (transient tachypnea of the newborn, RDS, or pneumothorax) as compared with infants with Ile/Thr (9.6%) or Ile/Ile (8.0%) genotypes, but the number of such infants was small, and this difference was not statistically significant ($P = 0.712$). Infants with respiratory distress had lower SP-B levels than controls ($P < 0.001$) with mean/median (SE) SP-B levels of 38.3/26.0 (7.6) and 63.8/56.0 (2.6), respectively (see **Supplementary Figure S1** online).

Linear regression analyses, taking into account the effects of multiple variables simultaneously, showed that the homozygosity

Table 1. The relative SP-B protein levels in amniotic fluid among the *SFTPB* genotype groups, showing statistically significant correlation between the genotypes and SP-B levels for rs1130866 but not for the other *SFTPB* polymorphisms

Polymorphism	Genotype	*n*	Mean	ANOVA *P* value	Median	SE	Kruskal–Wallis *P* value
rs3024791 (G/A at -384)	AA	1	65.00	0.531	65	0	0.594
	AG	40	55.20		51.5	5.49	
	GG	193	62.96		54	2.92	
rs2077079 (C/A at -18)	AA	102	60.33	0.228	53.5	3.97	0.295
	AC	117	59.66		52	3.60	
	CC	25	73.40		57	8.58	
rs1130866 (Ile131Thr)	Ile/Ile (TT)	75	61.44	0.013*	53	4.33	0.016*
	Ile/Thr (CT)	125	66.44		61	3.67	
	Thr/Thr (CC)	48	49.13		41	5.40	
Intron 4 length variation	Invar/Invar[a]	192	61.66	0.952	53	2.94	0.926
	Invar/Del[b]	51	60.18		59	4.85	
	Del/Del	2	47.00		47	1.00	

ANOVA, analysis of variance; SP-B, surfactant protein B.

[a]Invar, invariant allele (wild type). [b]Del, deletion variant allele. *Significant at $P < 0.05$.

of the Ile131Thr Thr allele (i.e., a recessive inheritance model where Thr/Thr group was compared against the combined Ile/Thr and Ile/Ile group), along with two other covariates gestational age (P = 0.001) and respiratory distress (P = 0.001), was a significant covariate for SP-B levels in amniotic fluid (P = 0.012). The other *SFTPB* genotypes and other factors (sex and birth weight) did not explain the SP-B levels, nor did they modify the effect of the *SFTPB* Thr/Thr genotype in adjusted models (data not shown).

SP-B N-Glycosylation and Secretion Kinetics

Both Chinese hamster ovary (CHO) cell lines that were generated to express one of the two human *SFTPB* Ile131Thr allelic variants showed robust proSP-B production and secretion in the culture medium. A prominent band of ~42 kDa corresponding to proSP-B was readily detectable in cell lysates and media (see **Supplementary Figure S2** online). Both recombinant proteins, proSP-B/Ile and proSP-B/Thr, were sensitive to peptide-N-glycosidase F due to COOH-terminal glycosylation at Asn311. However, the size shift was larger for proSP-B/Thr, consistent with the presence of an additional oligosaccharide in its NH_2 terminus at Asn129 that is absent in proSP-B/Ile (see **Supplementary Figure S2** online). These results provide evidence for allele-specific differences in the N-glycosylation of the proSP-B in CHO cells. This allele-specific glycosylation pattern is also seen in human lungs (15).

In pulse-chase labeling experiments with six paired replicates, proSP-B accumulation was detected in the medium in a time-dependent manner lagging ~30–60 min behind detection in the cell lysate (**Figure 1a**). The midpoint times of secretion, illustrated by the V50 values, were 51.9 ± 1.3 and 58.2 ± 1.2 min for proSP-B/Ile and proSP-B/Thr, respectively (**Figure 1b**). The results of the pulse-chase labeling experiments demonstrate that in CHO cells stably expressing proSP-B, the accumulation of NH_2-terminal N-glycosylated proSP-B/Thr in the culture medium was delayed by ~12% as compared with nonglycosylated proSP-B/Ile (P = 0.0012).

Allelic Expression Imbalance Assay

To determine whether the observed differences in the previous experiments arose transcriptionally or posttranscriptionally, we performed an allelic expression imbalance (AEI) assay using RNA from Ile131Thr heterozygous human adult lung tissue samples (n = 8). The expression ratio of the Ile and Thr variant mRNAs (multiple replicates), normalized by the reference sample ratio (plasmid DNA, 50:50), was 0.948 ± 0.111 (mean ± SD; range 0.792–1.165), which did not differ from the expected value of 1.0 (P = 0.229) (see **Supplementary Figure S3** online). This indicated equal mRNA expression and lack of AEI between the two variants.

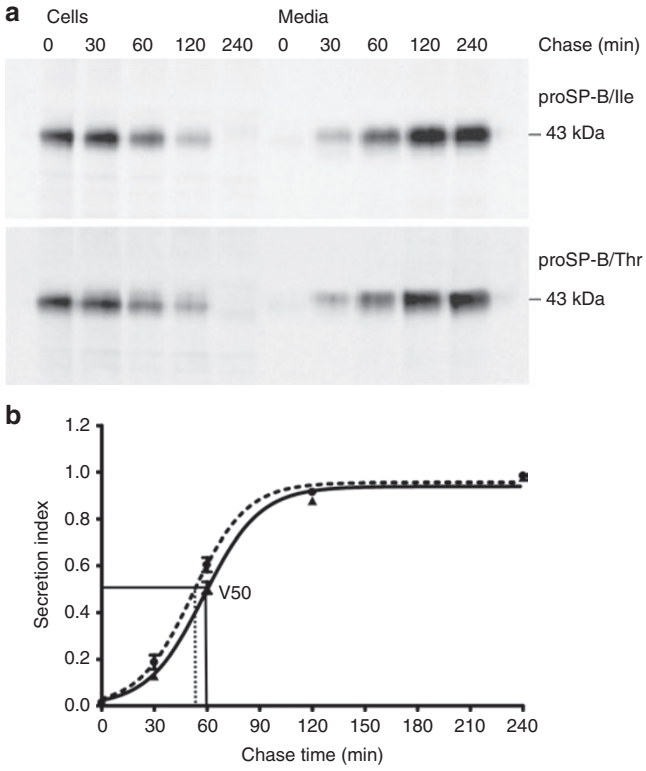

Figure 1. Allele-specific human proSP-B secretion kinetics in transfected Chinese hamster ovary (CHO) cells. Cells were pulse labeled for 30 min and chased for 0, 30, 60, 120, and 240 min. Both cells and media were harvested and proSP-B was recovered by immunoprecipitation. Representative phosphorimages are shown for both proSP-B Ile and Thr variants. (**a**) No proSP-B was seen in the medium until at the 30-min chase time point. Over time, proSP-B accumulated in the medium and the newly synthesized proSP-B was completely secreted from the cells within 240 min. (**b**) For curve fitting, the chase time was plotted against the secretion index. The secretion index, which describes the proportion of labeled proSP-B in the medium out of total labeled proSP-B, was calculated by dividing the trace quantity value of proSP-B in the medium by the sum of the corresponding values in the medium and cells. The data represent the mean ± SE from six independent experiments; black circles indicate proSP-B/Ile and black triangles indicate proSP-B/Thr. The value at 240-min time point was fixed to 1. The data were fitted to a Boltzmann sigmoidal model; dashed line and continuous line indicates proSP-B/Ile and proSP-B/Thr, respectively. V50 values, which illustrate the midpoint of secretion, were 51.9 ± 1.3 and 58.2 ± 1.2 min for proSP-B/Ile and proSP-B/Thr, respectively. Glycosylated proSP-B/Thr was secreted from CHO cells at a significantly lower rate than that of proSP-B/Ile (P = 0.0012). SP-B, surfactant protein B.

DISCUSSION

When studying complex traits, it is challenging to identify the causal risk variants and demonstrate the function of the implicated polymorphisms. Consequently, converting the results of both candidate gene and genome-wide association studies into precise molecular mechanisms and clinical applications is a slow process. The aim of this study was to perform molecular phenotyping for providing evidence for a common variant of *SFTPB* in disease predisposition. RDS presents a transient developmental deficiency in alveolar surfactant components shortly after premature birth (29). We and others have previously reported that there are associations of *SFTPB* polymorphisms with RDS and other lung diseases (15–21,25–27). We have also hypothesized a causal role for the Ile131Thr substitution, which selectively alters Asn129 N-glycosylation of the SP-B proprotein (15,17). In this study, this glycosylation showed delayed proSP-B secretion *in vitro* and an association with decreased extracellular SP-B concentration *in vivo*.

The analyses between amniotic fluid SP-B concentration and *SFTPB* polymorphisms revealed an associating polymorphism: Ile131Thr (rs1130866). In individuals with the homozygous Thr/Thr fetal genotype, we detected relatively lower levels of SP-B protein in amniotic fluid at the time of birth. SP-B production is well known to increase from preterm toward term gestation resulting in a rise in the amniotic fluid SP-B concentrations (30,31). Given this, our finding is of specific interest considering that the focus of these analyses was to investigate the term pregnancies without specific clinical complications and which were expected to show high SP-B concentrations. It remains to be explored whether extending these analyses to preterm deliveries with lower amniotic fluid SP-B concentrations would yield a stronger correlation between the genotype and the protein level.

To provide a more mechanistic explanation for these findings, we investigated the effect of the N-glycosylation status at Asn129 on SP-B secretion kinetics using commercially available CHO cells for generating stably transfected allele-specific proSP-B variant cell lines. Glycosylated proSP-B/Thr was secreted from the CHO cells at a significantly lower rate than that of proSP-B/Ile. This could be associated with the decreased levels of SP-B that were observed in the amniotic fluid samples with fetal Thr/Thr genotypes. The fact that the *in vitro* observations were obtained in a cell model lacking a regulated secretory pathway and cell type–specific processing steps implies that the underlying diverging molecular events occur during very early steps. The NH_2-terminal peptide containing the Asn129 site is removed from proSP-B very soon posttranslationally already in the ER (11). This step is not cell specific, as it has also been shown to occur in constantly secreting cells normally not expressing SP-B (16). As a whole, these data are consistent with the view that in type 2 cells, the relevant determinants directing the differential fate of the mature SP-B molecules derived from the variant proSP-B peptides should act very soon posttranslationally. Therefore, the CHO cell model could provide supporting mechanistic evidence for the functional role of the glycosylation status of Asn129 present in the Thr variant. However, proSP-B is not secreted from type 2 cells and therefore it is not straightforward to generalize the data from CHO cells to SP-B secretion *in vivo*. Nevertheless, these *in vitro* results supplemented with *in vivo* data using the amniotic fluid samples point to the same direction, i.e. delay in the intracellular events in the processing, sorting or secretion of the glycosylated allelic variant. Together these findings suggest a causal role for the single-nucleotide polymorphism rs1130866 *in vivo*. However, it remains in question whether these results can be repeated on alveolar type 2 cells that are capable of handling all the steps involved in the processing and packaging of SP-B *in vivo*.

To exclude the possibility that the Thr variant–associated decreased extracellular SP-B concentration in amniotic fluid and the reduced secretion rate in transfected cells was due to altered transcriptional activity of the variant, we performed an AEI assay in which the allelic ratios of the mRNA variants transcribed in adult human lungs were analyzed. The ratios for the Ile and Thr allele products did not deviate significantly from 1. This suggests that the intrinsic transcription levels of the Ile131Thr variants are equal and the differences in protein levels that we observed are not due to the allele-specific preferences in mRNA expression.

N-glycosylation is known to have a definitive role in governing protein secretion, and it is thought to enhance the folding, trafficking, and stability of glycoproteins by targeting the use of ER folding cycles via chaperones (32). Defective glycosylation caused by mutations typically leads to mislocalization, ER retention, and ER-associated protein degradation, whereas glycosylation sites have also been shown to facilitate improved protein trafficking (33–36). Glycosylation of proSP-B represents a different paradigm. Instead of an infrequent mutation resulting in disease, Ile131Thr results from a single-nucleotide polymorphism in which the potentially unfavorable glycosylated Thr variant allele occurs at a high frequency in the population. Both of these variants allow for processing of fully functional mature protein. Therefore, although the glycosylated Thr variant of SP-B may have different properties compared with the wild-type Ile variant, it is apparently, e.g., not too aberrant to be recognized as a misfolded protein in the ER and destined for proteasomal degradation. It is plausible that the proSP-B Thr variant undergoes slight ER retention, possibly through a prolonged interaction with an ER chaperone protein that assists in folding. This would be adequate for explaining the subtle delay in secretion observed in our study. Of note, when two N-glycosylation sites of the human δ-opioid receptor were mutated, enhanced export of the non-N-glycosylated receptors from the ER was observed (37). This effect on kinetics by glycosylation is similar to what we observed in our study.

As for possible limitations in this study, western blot quantitation with infrared fluorescence is suboptimal in its accuracy for diagnostics, but yet it can be considered adequate for correlation studies not aiming at precise absolute quantitation. Western blot–based quantitation enabled us to distinguish between all proSP-B intermediates and mature SP-B and helped us to overcome the background due to nonspecific binding, typical of enzyme-linked immunosorbent assay–based SP-B quantitation method. Protein quantitation by western immunoblotting with an antibody directly labeled with a near-infrared dye has been shown to be sensitive, linear, and reproducibly quantifiable over a wide range of concentrations (38,39). In addition, the total volume of amniotic fluid varies highly between pregnancies causing intrinsic variation in protein concentrations. This is evident as high variation in SP-B levels in the unaffected subjects in our study. Despite this basal background variation, statistically significant correlations were observed between the protein levels and the genotype. Taking into account the limitations of the quantitation method and variability in amniotic fluid volumes, these analyses are not expected nor meant to provide us with diagnostic value.

In summary, we observed *in vivo* lower concentrations of mature SP-B secreted from fetal lungs into amniotic fluid in rs1130866 Thr/Thr homozygotes compared with the other genotypes. These findings were further supported by *in vitro*

experiments showing delayed secretion of the glycosylated proSP-B/Thr variant compared with the nonglycosylated proSP-B/Ile variant in transfected cells. No allelic imbalance was evident in heterozygous Ile/Thr lung tissue samples, suggesting a posttranscriptional mechanism. Together these data suggest that the presence of the NH_2-terminal N-glycosylation site due to the amino acid substitution at proSP-B position 131 may be a genetic determinant of delayed passage of the protein through the secretory pathway and decreased extracellular SP-B concentrations. This effect is not substantial enough to decrease the SP-B protein contents below the critical level, which is a decrease by >50% of the normal alveolar SP-B content (28), for normal pulmonary function under stable conditions. However, even a smaller decrease could serve as a trigger for transient respiratory distress during the critical first few minutes of life after premature birth, when maintenance of adequate alveolar volumes for normal efficient breathing and gas exchange needs to start immediately with no delay in the optimal secretion of surfactant constituents. As a conclusion, our study provides mechanistic evidence to support a causal role of the *SFTPB* Ile131Thr polymorphism, which has previously been shown in several studies to associate with genetic susceptibility to pulmonary outcomes, such as RDS.

METHODS

Detailed description of the materials and methods are available in the **Supplementary Data** online.

Sample Collection and Study Population

Sample pairs of amniotic fluid and umbilical cord blood (n = 251) were obtained from elective cesarean section deliveries of term singleton pregnancies with gestation ranging from 37 to 40 wk. The characteristics of the study population are shown in **Table 2**. Detailed diagnostic criteria for respiratory symptoms are presented in the **Supplementary Data** online. Adult human lung tissue was obtained from patients undergoing lung surgery. Informed consent was obtained from all the subjects. The study was approved by the Ethics Committee of the Oulu University Hospital.

Amniotic Fluid Sample Preparation and Western Analysis

Proteins from amniotic fluid samples were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A pooled amniotic fluid sample known to contain high levels of SP-B was used as a standardized reference in each western blot. The western quantitation assay was explored for reproducibility, linearity, level of detection, and sensitivity as described in the **Supplementary Data** online.

Genotyping of *SFTPB* Gene Polymorphisms

Genotyping of *SFTPB* Ile131Thr (rs1130866) polymorphism and Δi4 was performed as described previously (17). For C/A(−18) (rs2077079) and A/G(−384) (rs3024791), primer pairs and restriction enzymes were designed for polymerase chain reaction and restriction fragment length polymorphism analyses as described in the **Supplementary Data** online.

Table 2. The characteristics of the study population used for amniotic fluid SP-B quantitation

			Gestational age (wk + d)		
	n	Male/female	Mean	Median	Range
All infants	251	137/114	38 + 6	39 + 0	37 + 0 to 40 + 6
TTN	19	12/7	38 + 4	38 + 4	37 + 1 to 40 + 0
RDS	1	0/1	37 + 5	37 + 5	37 + 5
Pneumothorax	3	2/1	38 + 6	38 + 6	38 + 3 to 39 + 3

RDS, respiratory distress syndrome; SP-B, surfactant protein B; TTN, transient tachypnea of the newborn.

Reverse Transcription PCR, Construction of Plasmids, and Generation of Stably Transfected ProSP-B–Expressing CHO Cells

RNA from human lung tissue heterozygous for *SFTPB* Ile131Thr was reverse transcribed to cDNA and amplified by polymerase chain reaction. The 1,146 bp reverse transcriptase-polymerase chain reaction products were subcloned, and the resulting expression constructs containing the Ile or Thr variant cDNA were transfected into Flp-In-CHO cells (Invitrogen, Carlsbad, CA). Cells were cultured and the SP-B–expressing clones were selected. Justification for the use of CHO cells, instead of using cells such as alveolar type 2 cells, is described in detail in the **Supplementary Data** online.

ProSP-B Detection in CHO Cells and Carbohydrate Removal With Peptide-N-glycosidase F

Cells and culture media were assayed for SP-B/Ile and SP-B/Thr expression and secretion by western blot analysis. Peptide-N-glycosidase F digestion was performed to confirm the presence of asparagine-linked oligosaccharides in the expressed proSP-B variants.

Pulse-Chase Labeling, Immunoprecipitation, and Immuno-blotting

CHO cells stably expressing either proSP-B/Ile or proSP-B/Thr were transferred from Ham's F-12 to Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO). Nontransfected CHO cells were used as control for absence of SP-B. After a 30-min pulse, both cells and media were harvested at intervals of 0, 30, 60, 120, and 240 min during the 240-min chase. Radiolabeled CHO cell pellets were lysed, and both lysates and media were used for immunoprecipitation to analyze the kinetics of secretion.

AEI Assay

cDNA from adult human lung tissue samples (n = 8) heterozygous for the *SFTPB* Ile131Thr polymorphism were tested for AEI using SNaPshot assay (Applied Biosystems, Foster City, CA).

Statistical Analyses

Statistical analyses were performed using PASW Statistics 18 Data Editor (LEAD Technologies, Charlotte, NC). Parametric (ANOVA) and nonparametric (Kruskal–Wallis) tests were used for analyzing the correlation between SP-B protein levels and genotypes. Linear regression was used for multivariate analysis and one-sample t-test for AEI analysis. A P value of <0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

ACKNOWLEDGMENTS

We thank Maarit Haarala, Mirkka Ovaska, Panu Taskinen, and Riitta Vikeväinen for their technical assistance and sample collection and Maritta Pietilä for her methodological advice.

STATEMENT OF FINANCIAL SUPPORT

This work was supported by the Academy of Finland (R.H. and M.H.), Foundation of Pediatric Research in Finland (R.H.), Päivikki and Sakari Sohlberg Foundation (R.H.), Sigrid Juselius Foundation (M.H.), and HL059959 (S.H.G.) from the National Institutes of Health (Bethesda, MD).

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