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Surfactant Protein-C Promoter Variants Associated with Neonatal Respiratory Distress Syndrome Reduce Transcription

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Abstract

Dominant mutations in coding regions of the surfactant protein-C gene (*SFTPC*) cause respiratory distress syndrome (RDS) in infants. However, the contribution of variants in noncoding regions of *SFTPC* to pulmonary phenotypes is unknown. Using a case-control group of infants ≥ 34 weeks gestation ($n=538$), we used complete resequencing of *SFTPC* and its promoter, genotyping, and logistic regression to identify 80 single nucleotide polymorphisms (SNPs). Three promoter SNPs were statistically associated with neonatal RDS among European descent infants. To assess the transcriptional effects of these three promoter SNPs, we selectively mutated the *SFTPC* promoter and performed transient transfection using MLE-15 cells and a firefly luciferase reporter vector. Each promoter SNP decreased *SFTPC* transcription. The combination of two variants in high linkage disequilibrium also decreased *SFTPC* transcription. *In silico* evaluation of transcription factor binding demonstrated that the rare allele at g.-1167 disrupts a SOX (SRY-related high mobility group box) consensus motif and introduces a GATA-1 site, at g.-2385 removes a MZF-1 (myeloid zinc finger) binding site, and at g.-1647 removes a potential methylation site. This combined statistical, *in vitro*, and *in silico* approach suggests that reduced *SFTPC* transcription contributes to the genetic risk for neonatal RDS in developmentally susceptible infants.

Introduction

Pulmonary surfactant is a phospholipid and protein complex that is synthesized by alveolar type II cells and maintains alveolar expansion at end expiration. Developmentally regulated deficiency of surfactant due to immaturity of alveolar type II cells in prematurely born infants disrupts fetal-neonatal pulmonary transition by causing alveolar collapse at end expiration and neonatal respiratory distress syndrome (RDS) (1). Neonatal RDS may also result from genetic disruption of pulmonary surfactant metabolism as suggested by heritability estimates (0.2–0.8 in twin studies), by rare, lethal mutations in genes encoding surfactant protein-B, (*SFTPB*, gene ID 6439, MIM 178640), surfactant protein-C (*SFTPC*,

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gene ID 6440, MIM 178620), and the ATP-binding cassette, subfamily A, member 3 (*ABCA3*, gene ID 27410, MIM 601615), and by genotyping studies of common, non-synonymous variants in *SFTPB* and *SFTPC* (2-8). Furthermore, recent studies report an increased risk of neonatal RDS likely attributable to surfactant deficiency in late preterm infants (≥ 34 weeks gestation)(9-11).

SFTPC encodes surfactant protein-C (SP-C), a lung-specific extremely hydrophobic peptide that spans the phospholipid bilayer of pulmonary surfactant and contributes to maintenance of alveolar expansion at end expiration (12). *SFTPC* is genomically small (3.5 kb with a 3.7kb promoter), located on human chromosome 8, and directs the synthesis of an alternatively spliced 191 or 197 amino acid proprotein (proSP-C) that undergoes sequential proteolytic cleavages to yield the 35 amino acid mature SP-C peptide (12,13). Studies performed on fetal human lung tissue demonstrate developmental regulation of *SFTPC* with mRNA detected by 13-15 weeks gestation (14,15) and pro-SPC protein by 12-16 weeks (16); SP-C expression increases with advancing gestational age to approximately 15% of adult levels by 24 weeks gestation (15).

Dominantly expressed, rare, exonic mutations in *SFTPC* cause respiratory dysfunction of varying severity and age of onset among infants, children, and adults which is thought to result from aggregation of misfolded or misrouted proSP-C peptides that exceed the capacity of cell stress response pathways to maintain cellular homeostasis (6,17-20). Furthermore, two common non-synonymous variants that encode an asparagine for threonine substitution at codon 138 (p.T138N, rs4715) and an asparagine for serine substitution at codon 186 (p.S186N, rs1124) have been statistically associated with RDS among premature infants <34 weeks gestation (21). However, no comprehensive sequence analyses of the contribution of rare variants in *SFTPC* to RDS have been performed.

To investigate the contribution of rare and/or non-coding region variants in *SFTPC* to neonatal RDS in term or late preterm infants, we used complete resequencing or genotyping of *SFTPC* in newborns ≥ 34 weeks gestation with and without RDS to identify variants statistically associated with neonatal RDS, *in silico* evaluation of transcriptional function, and transfection of a murine pulmonary epithelial cell line to confirm functional significance of statistically associated variants.

Materials and Methods

Case-Control Study

We recruited 184 consecutive term and late preterm newborn infants (≥ 34 weeks gestation) with RDS and, separately, a control group of 354 infants without respiratory symptoms who were referred to the Division of Newborn Medicine at St. Louis Children's Hospital for clinical care (8) (Table 1). To standardize case phenotype, RDS was defined as the need for supplemental oxygen ($FiO_2 \geq 0.3$) for >24 hours, a chest radiograph consistent with neonatal RDS, and the need for continuous positive airway pressure or mechanical ventilation within the first 48 hours of life. Controls (CON) were term or late preterm infants without respiratory symptoms. Gestational age for each infant was assigned based on best obstetrical estimate. We excluded infants with congenital anomalies that could contribute to respiratory distress, known genetic causes of respiratory insufficiency (e.g. *SFTPB* or *ABCA3* deficiency), culture positive sepsis, chromosomal anomalies, late onset RDS (>48 hours), and transient tachypnea of the newborn (TTN) which resolved within 24 hours of life. A single twin from each pair of monozygotic twins was included. The designation of RDS or control status was assigned prior to any genetic studies and without knowledge of *SFTPC* genotype. We obtained informed consent from parents of all infants.

We performed complete *SFTPC* resequencing, including the promoter and intervening introns, for 269 infants (92 RDS, 177 CON). Interim analyses revealed overrepresentation in the cases of the minor allele at 3 *SFTPC* promoter sites, so we performed genotyping of these three variants in an additional 269 infants (92 RDS, 177 CON) using 5' nuclease assays. This study was approved by the Human Research Protection Office of Washington University School of Medicine.

DNA Isolation, Sequencing, and Genotyping

We isolated DNA from blood using Puregene DNA isolation kit (Qiagen). We bidirectionally sequenced all translated exons, introns, and promoter region of *SFTPC* (~6kb total) from 269 infants as previously described (22). We used Phred, Phrap, PolyPhred, and Consed (<http://www.phrap.org/phredphrapconsed.html>) to identify and annotate single nucleotide polymorphisms (SNPs) in sequencing chromatograms and Prettybase (<http://pga.mbt.washington.edu>) to extract a final file with genotypes. We obtained human *SFTPC* reference sequence from Alamut® (Version 1.5, Interactive Biosoftware, Inc. Rouen, France). We used 5' nuclease assays (Taqman®, Applied Biosystems) and the ABI 7500 FAST Real Time PCR system to genotype the three promoter sites identified in interim analyses for an additional 269 infants.

Statistical Methods

After anonymously linking individual genotype and phenotype information, we used SAS (Version 9.1.3, SAS Institute, Cary, NC) to perform interim analyses using race-stratified Fisher's exact test to determine the association of *SFTPC* variants with risk of RDS. For our final analyses, we performed race-stratified logistic regression controlling for the effects of estimated gestational age and gender (23) to determine the association of the identified promoter variants with risk for neonatal RDS. To control for inflated type 1 error due to multiple association tests required for each of the *SFTPC* variants ($n=80$), we used a modified false discovery rate (FDR) approach (24) based on the number of effective tests. Specifically, we considered the gene variants within one haplotype block as one effective test, and we determined an adjusted p -value using the formula: $p=0.05/(1+1/2+1/3+1/4+\dots+1/n)$, where n is number of effective tests. Based on this calculation, we determined a p -value of 0.01 as a criterion for statistical significance.

In silico Functional Analysis of SNPs

To identify transcription factor binding motifs and determine the predicted functional significance of promoter region variants, we used TFSEARCH95 (www.cbrc.jp), an application that predicts transcription factor consensus sites based on a weighted matrix.

In vitro Functional Analysis of SNPs

We created the reporter plasmid *SFTPC_WT_luc* by subcloning the 3.7 kb human *SFTPC* promoter (kind gift of J. Whitsett) into a HindIII site of the firefly luciferase reporter vector pGL4.10(luc2) (Promega, Madison, WI). We generated the 3 *SFTPC* promoter variants associated with RDS (*SFTPC_1167G_luc*, *SFTPC_1647A_luc* and *SFTPC_2385T_luc*) and a combination of variants in high linkage disequilibrium (LD) (*SFTPC_1647_2385*) by site-directed mutagenesis (Stratagene, La Jolla, CA) and confirmed all construct sequences by DNA sequencing. To evaluate the effect of each promoter variant or combination on transcription, we used Fugene 6 (Roche Applied Science, Indianapolis, IN) to transiently transfect MLE-15 cells (kind gift of J. Whitsett), an immortalized mouse lung epithelial cell line with functional and morphological characteristics similar to pulmonary type II epithelial cells (25,26). Twenty-four hours after seeding MLE-15 cells (1.5×10^5 cells/well in 12-well plates) in HITES medium (RPMI 1640 medium with 2% FBS, supplemented with 5 μ g/ml

insulin, 10 µg/ml transferrin, 5 ng/ml sodium selenite, 10 nM β-estradiol, 10 nM hydrocortisone, 2mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin), we co-transfected each *SFTPC* reporter construct with pGL4.74 (hRluc-TK) vector (Promega) with 6 replicates for each construct. After 48 hours, we lysed the cells and measured luciferase activities using the dual-luciferase reporter assay system (Promega). We normalized relative light units of firefly luciferase activity with Renilla luciferase activity and performed all transfections three times. We compared normalized luciferase activity between the wild type promoter and each construct using Student's paired t-tests.

Results

SFTPC Variants Associated with Neonatal RDS

We identified 80 variants within *SFTPC* and its promoter with complete resequencing of 269 infants (Table S1, <http://links.lww.com/PDR/XXX>). Analyses using Fisher's exact test revealed overrepresentation in cases of the minor alleles at genomic positions g.-1647(A) and g.-2385(T) among infants of European descent with RDS and g.-1167(G) among infants of African descent with RDS (Table 2). The alleles at g.-1647 and g.-2385 are in strong LD ($r^2=0.75$ for African descent infants, $r^2=0.84$ for European descent infants). Race stratified logistic regression models that included gestational age and gender (23) performed on the final cohort of 538 infants revealed overrepresentation of the minor alleles of all three promoter variants (g.-1167, g.-1647, and g.-2385) among infants of European descent with RDS (Table 3). There was a trend toward overrepresentation of the minor allele of g.-1167(G) among infants of African descent with RDS, however, this trend did not achieve statistical significance. The variants at g.-1647 and g.-2385 were not associated with neonatal RDS in the African descent infants. Additionally, no deleterious variants in *SFTPB* or *ABCA3* were identified in any of the RDS infants.

Two promoter region variants are within transcription factor binding sites

Using TFSEARCH95, we found that the minor allele at g.-1167 disrupts a SOX (SRY-related high mobility group box) consensus motif and introduces a GATA-1 site, at g.-2385 removes a myeloid zinc finger (MZF-1) binding site, and at g.-1647 removes a potential methylation site. These findings suggest that 2 of the 3 statistically identified *SFTPC* promoter variants could regulate transcription by altering transcription factor binding and the third by altering methylation.

Promoter region variants decrease transcription

Each of the three *SFTPC* variants significantly decreased transcription of the *SFTPC* promoter as measured by decreased luciferase reporter activity relative to the wild type construct (Figure 1). The construct for g.-2385 reduced *SFTPC* promoter transcription by approximately 13% ($p=0.03$), g.-1647 by approximately 19% ($p=0.005$), and g.-1167 by approximately 59% ($p=1.7\times 10^{-6}$). The construct with the two variants in high LD (g.-1647+g.-2385) reduced transcription by approximately 56% ($p=0.003$). We transfected each promoter construct three times with similar findings. These findings suggest that the minor alleles at each of the 3 statistically identified *SFTPC* promoter variants and the combination of g.-2385 and g.-1647 reduce transcription.

Discussion

We found that three *SFTPC* promoter SNPs were statistically overrepresented among infants of European descent with RDS, were predicted by *in silico* testing to impact transcription, and functionally decreased transcription in a murine alveolar epithelial cell line. In contrast to exonic *SFTPC* mutations that result in pulmonary disease in children and adults due to

protein misfolding (6,17,18), these findings suggest that a proportion of the risk for neonatal RDS is attributable to transcriptional regulation of *SFTPC*. Population-based analyses of these variants are underway to determine their attributable risk to RDS.

In the absence of an accompanying change in protein structure, mutations in the regulatory regions of genes may be overlooked as a mechanism for phenotypic variation and generally require functional analysis of gene expression, such as transient transfection. The phenotypic variation associated with these *cis*-regulatory mutations is presumed to be due to changes in transcript abundance. In a review of 107 human genes with identified *cis*-regulatory polymorphisms, 63% demonstrated allelic differences in expression of two-fold or greater with transient transfection (27). For example, two common variants within the promoter of the Surfactant Protein-B gene have been associated with decreased gene transcription. The g.-18 C>A variant is associated with decreased Sp-1 binding and lower concentrations of Surfactant Protein-B in the bronchoalveolar lavage fluid of healthy adult volunteers (28) the g.-384 G>A variant impairs binding of TTF-1 (thyroid transcription factor-1) and reduces transcriptional activity in H441 cells (29).

Like exonic *SFTPC* mutations, these promoter variants appear to act in a dominant fashion, as the presence of a single copy of the minor/ risk allele increases the risk for RDS among European descent infants and was sufficient to decrease gene expression *in vitro*. A single infant was homozygous for the rare variant at g.-2385; otherwise all individuals were heterozygous. *Cis*-regulatory mutations may act in a co-dominant fashion due to allele-specific transcript abundance, whereas mutations in the coding sequence are more likely to act in a recessive fashion. Due to the nature of transcriptional regulation, the effect of *cis*-regulatory mutations may be limited temporally or spatially depending on the specific regulatory site affected (30). For these three promoter variants in *SFTPC*, the context-dependent regulation of gene expression may have important implications for the developmentally susceptible infant who requires a threshold of *SFTPC* expression for successful fetal-neonatal pulmonary transition. Although full-term mice with genetically abrogated *SFTPC* expression are viable at birth, have normal pulmonary function, exhibit normal lung morphology, and have similar rates of surfactant synthesis, secretion, and pool size, their pulmonary surfactant is functionally deficient due to reduced hysteresivity and instability at low bubble volumes (31). When these mice are subjected to a significant pulmonary insult (e.g. intratracheal bleomycin), they exhibit greater inflammation, increased collagen accumulation, and delayed recovery when compared to wild-type mice (32). Surfactant from SP-B +/- SP-C +/- mice exhibit microbubble stability by bubble surfactometer when compared to surfactant from SP-B +/- SP-C -/- mice indicating that SP-C plays a role in stabilization of phospholipid film (33).

To our knowledge, the only other study to evaluate a statistical association of *SFTPC* variants and neonatal RDS used genotyping strategies and found that two common non-synonymous SNPs, c.413C>A (p.T138N, rs4715, minor allele frequency approximately 0.20) and c.557G>A (p.S186N, rs1124, minor allele frequency approximately 0.22) were overrepresented among Finnish preterm infants with RDS (21). We did not find these associations in our study, perhaps due to the differences in gestational ages (38.2 (U.S.) vs. 28.6 weeks (Finnish)) or differences in ethnicities between the two populations. However, the range of gestational ages in our cohort was more limited than that of the Finnish cohort, and these polymorphisms may be more influential during earlier periods of development.

When we performed race stratified analyses, both gender and gestational age were incorporated as covariates into the final regression model. Gender was a significant predictor for RDS risk among African descent infants, but not for European descent infants in our study; estimated gestational age was an independent predictor of RDS risk for both races.

We also considered the possibility of an inflated type I error rate and used a FDR approach to correct for multiple tests. The promoter variants we found to be statistically associated with RDS among infants of European descent approximated the FDR p-value and were further validated in a transient transfection system to decrease transcription of a reporter gene. It is also possible that the African descent cohort may have been underpowered to detect associations with the 3 promoter SNPs identified in European descent infants due to the limited number of African descent infants with RDS. This disparity reflects what we observe clinically as there are fewer infants of African descent with RDS (34), and our study enrolled infants consecutively. Finally, the frequencies of the 3 transcriptionally active promoter SNPs were too low to provide statistical power to assess associations with disease severity.

Taken together, these data suggest that *SFTPC* promoter variants increase the risk for neonatal RDS in late preterm and term infants by reducing *SFTPC* transcription.

This combined statistical, *in silico*, and *in vitro* approach suggests that reduced *SFTPC* transcription contributes to the genetic risk for neonatal RDS in developmentally susceptible infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SNP	Single nucleotide polymorphism
SFTPB	Surfactant Protein-B Gene
SFTPC	Surfactant Protein-C Gene
SP-C	Surfactant Protein-C
MLE	Mouse lung epithelium
FDR	False discovery rate
LD	Linkage Disequilibrium

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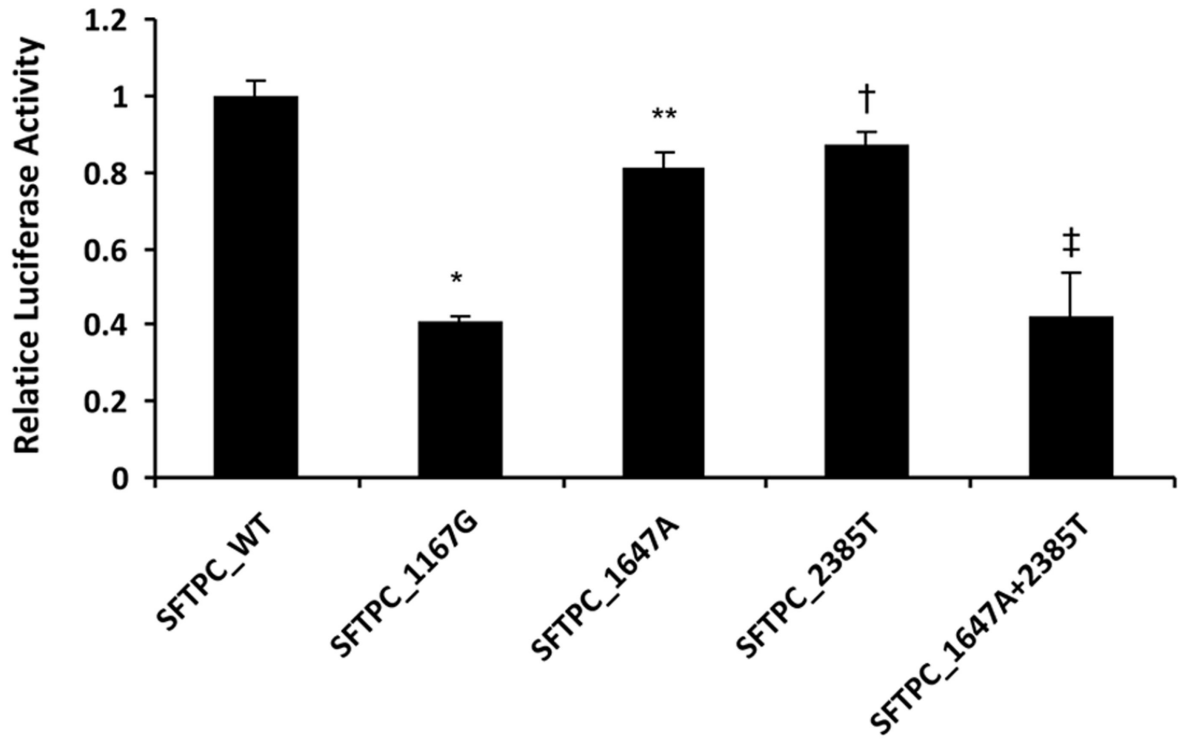


Figure 1. Three individual promoter variants: SFTPC_1167G, SFTPC_1647A and SFTPC_2385T and the combination SFTPC_1647A_2385T decrease transcriptional activity of human *SFTPC* promoter relative to the wildtype promoter

Data are mean ± S.D. from 1 representative experiment (n=6 replicates per construct). The experiments were performed 3 times with similar results. * p= 1.7×10^{-6} ** p= 0.0045 † p= 0.030 ‡ p=0.0026

Table 1

Characteristics of Case-Control Group (538)

African-Descent (AD)	RDS	CON	p-value
Total[†]	40	215	
Sex[†]			
Female	11	108	0.009
Male	29	107	
Birth Weight (Mean ±SD)	3.00 (±0.97)	3.11 (±0.47)	0.272
EGA (wks)	37.8 (±2.6)	38.9 (±1.7)	0.0006
Route of Delivery[†]			
Vaginal	17	142	0.084
C-Section	17	73	
Unknown	6	0	
European-Descent (ED)	RDS	CON	p-value
Total[†]	144	139	
Sex[†]			
Female	58	63	0.403
Male	86	76	
Birth Weight (Mean ±SD)	3.10(± 0.65)	3.12 (±0.63)	0.787
EGA (wks)	37.2 (±2.1)	38.2 (±1.6)	<0.0001
Route of Delivery[†]			
Vaginal	62	74	0.115
C-Section	60	75	
Unknown	10	2	

[†]Number of infants

Table 2
Fisher's Exact Test: Genotype association of *SFTPC* variants with RDS

Race-stratified Fisher's exact analyses of the association of *SFTPC* variants with RDS. Three promoter variants were statistically associated with neonatal RDS: the minor alleles at g.-1647 and g.-2385 were overrepresented among infants of European descent with RDS and the minor allele at g.-1167 was overrepresented among infants of African descent with RDS.

European Descent (ED)				
	Genotype	CON	RDS	Fisher's
rs8192321	AA	82	62	0.140
(g.-1167)	AG	2	6	
rs8192337	GG	82	59	0.015
(g.-1647)	AG	0	5	
rs8192335	GG	85	63	0.016
(g.-2385)	GT	0	4	
	TT	0	1	
African Descent (AD)				
		CON	RDS	Fisher's
rs8192321	AA	89	20	0.093
(g.-1167)	AG	3	3	
rs8192337	GG	90	23	0.375
(g.-1647)	AG	1	1	
rs8192335	GG	90	23	0.375
(g.-2385)	GT	1	1	

Table 3
Logistic Regression: Race-specific genotype association of *SFTPC* promoter variants with RDS

Race-stratified logistic regression of the association of *SFTPC* variants with RDS. Estimated gestational age and gender were incorporated into the models as covariates. The minor alleles of three promoter variants were overrepresented at g.-1167, g.-1647 and g.-2385 among infants of European descent with RDS; there was a trend toward overrepresentation of the minor allele at g.-1167 among infants of African descent with RDS.

European Descent (ED)				
	CON	RDS	Regression	Odds Ratio (CI)
rs8192321	AA 132	129	0.019	4.14 (1.26-13.57)
(g.-1167)	AG 4	13		
rs8192337	GG 137	131	0.017	12.75 (1.58-103.15)
(g.-1647)	AG 1	11		
rs8192335	GG 136	132	0.016	13.0 (1.60-105.09)
(g.-2385)	GT 1	10		
	TT 0	1 [†]		
African Descent (AD)				
	CON	RDS	Regression	Odds Ratio (CI)
rs8192321	AA 207	35	0.058	3.68 (0.96-14.17)
(g.-1167)	AG 7	4		
rs8192337	GG 210	39	0.491	2.29 (0.22-24.07)
(g.-1647)	AG 3	1		
rs8192335	GG 210	38	0.417	2.78 (0.24-32.55)
(g.-2385)	GT 2	1		

[†]TT individual combined with GT group for statistical analyses