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Population and Disease-Based Prevalence of the Common Mutations Associated With Surfactant Deficiency

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ABSTRACT: The prevalence of the common mutations in the surfactant protein-B (121ins2), surfactant protein-C (I73T), and ATP-binding cassette member A3 (E292V) genes in population-based or case-control cohorts of newborn respiratory distress syndrome (RDS) is unknown. We determined the frequencies of these mutations in ethnically diverse population and disease-based cohorts using restriction enzyme analysis (121ins2 and E292V) and a 5′ nuclease assay (I73T) in DNA samples from population-based cohorts in Missouri, Norway, South Korea, and South Africa, and from a case–control cohort of newborns with and without RDS ($n = 420$). We resequenced the ATP-binding cassette member A3 gene (*ABCA3*) in E292V carriers and computationally inferred *ABCA3* haplotypes. The population-based frequencies of 121ins2, E292V, and I73T were rare (<0.4%). E292V was present in 3.8% of newborns with RDS, a 10-fold greater prevalence than in the Missouri cohort ($p < 0.001$). We did not identify other loss of function mutations in *ABCA3* among patients with E292V that would account for their RDS. E292V occurred on a unique haplotype that was derived from a recombination of two common *ABCA3* haplotypes. E292V was over-represented in newborns with RDS suggesting that E292V or its unique haplotype impart increased genetic risk for RDS. **(*Pediatr Res* 63: 645–649, 2008)**

The pulmonary surfactant is a phospholipid-protein complex synthesized in alveolar type II cells and necessary for maintaining alveolar expansion at end-expiration. The pulmonary surfactant metabolic cycle includes synthesis, trafficking, processing, secretion, and recycling and maintains alveolar homeostasis (1). In symptomatic newborn infants and older children, mutations in three genes of the surfactant synthetic pathway, surfactant proteins-B and -C (*SFTPB* and *SFTPC*) and the ATP-Binding Cassette member A3 (*ABCA3*), disrupt surfactant function and cause respiratory disease in newborns and older children. Surfactant proteins-B (SP-B) and -C (SP-C) are hydrophobic peptides within the surfactant phospholipid layer that contribute to surface activity (2), whereas the ATP-binding cassette protein A3 (ABCA3) likely transports lipids, such as phosphatidylcholine, cholesterol, sphingomyelin, and phosphatidylglycerol, into lamellar bodies where the surfactant complex is assembled, processed, and stored (3–6).

SP-B is a 79-amino acid, hydrophobic protein encoded by a 9.7 kb gene, *SFTPB*. Infants homozygous for recessive loss-of-function mutations in *SFTPB* develop respiratory failure shortly after birth that is fatal without lung transplantation (7). The *SFTPB* allele most commonly observed in infants with SP-B deficiency (>60% of mutated alleles) results from a frameshift at codon 121 (121ins2) and is rare: less than 1 per 1000 individuals in two different population-based cohorts in the United States (8–10).

SP-C is a 35 amino acid hydrophobic protein, encoded by a 3 kb gene, *SFTPC*. Known mutations in *SFTPC* are expressed in a dominant fashion and have been associated with respiratory distress and interstitial lung disease in newborns and older children (11,12). The most common *SFTPC* mutation is a single nucleotide transition that results in a threonine for isoleucine substitution at codon 73 (I73T) and is present in over 25% of the cases of SP-C associated disease (13).

ABCA3 is a 1704 amino acid protein, encoded by an 80 kb gene, *ABCA3*. Several autosomal recessive mutations in *ABCA3* have been linked to lethal surfactant deficiency in newborns (14,15) and to chronic respiratory insufficiency in older children (16). A missense mutation which introduces a valine for glutamic acid substitution at codon 292 (E292V), when associated with another mutation on the other *ABCA3* allele, has been described in older, unrelated children with chronic lung disease (16).

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Abbreviations: ABCA3, ATP-binding cassette, A3; **SNPs,** single nucleotide polymorphisms; **SP-B,** surfactant protein-B; **SP-C,** surfactant protein-C

Previously, we reported a prevalence of approximately 0.8/1000 for 121ins2 in an unselected cohort of anonymized bloodspots obtained from the Missouri Department of Health Newborn Screening Program (17). The frequencies of I73T and E292V in this population, and the frequencies of the three common surfactant pathway mutations in other geographically and ethnically diverse populations are unknown, as are their contributions to respiratory distress syndrome (RDS) in unselected populations of symptomatic newborns. To assess the population-attributable and disease-associated frequencies of these mutations that are rare in the general population but the most common of the disease-causing mutations in the three surfactant pathway genes, *SFTPB, SFTPC,* and *ABCA3*, we used high throughput molecular screening methods in four ethnically and geographically diverse population-based cohorts and a single institution referral-based case–control cohort.

METHODS

Population-based cohorts. Anonymized DNA specimens were obtained from the Newborn Metabolic Screening Programs of Missouri, Norway, South Korea, and South African blacks (supplemental material, online). Ethnic and gender data were only available for the Missouri cohort, which was 51% male, 49% female, and comprised of 77% white, 20% black, and 3% individuals from other racial groups.

Case–control cohort. We obtained DNA and clinical data from healthy term newborns as a control group (CON) and from newborns with RDS referred to the Division of Newborn Medicine at St. Louis Children's Hospital for clinical care or genetic screening (Table 1). We defined RDS as the need for supplemental oxygen, a chest radiograph consistent with RDS and the need for continuous positive airway pressure or mechanical ventilation within the first 24 h of life. Because each of 16 twin-pairs had concordant results, only one twin from each pair was included in the analyses.

The Human Research Protection Office at the Washington University Medical Center and the Institutional Review Board at the Missouri Department of Health and Senior Services and of the respective countries' newborn screening programs approved this study. Informed consent was obtained from the parents of the infants in the case–control cohort.

Surfactant protein-B. We used *SfuI* restriction enzyme analysis to screen for 121ins2 after amplifying a 354 base pair fragment of exon 4 as described previously (10,17) (supplemental material, online).

Surfactant protein-C. We used a 5′ nuclease assay (Taqman®, Applied Biosystems) and the ABI 7500 FAST Real Time PCR System to genotype the I73T mutation. The assay produced a 61 bp amplicon of exon 3, which included the thymine to cytosine transition responsible for I73T. Genomic DNA from individuals known to be heterozygous for I73T served as controls on each plate.

ATP-Binding Cassette member A3. We used *BsrG1* restriction enzyme analysis to screen for E292V after amplifying a 682 base pair nucleotide fragment of exon 8 that contained the adenine to thymine transversion (16)(supplemental material, online). To determine whether those newborns with E292V and RDS carried other unique, functionally disruptive mutations in *ABCA3,* we then amplified and sequenced 2 kb of the promoter region, the 30 coding and 2 noncoding exons, and splice site junctions of *ABCA3* for all 11 infants heterozygous for E292V and for 12 race and gestational age matched CON infants from the case–control cohort. Ethidium bromide agarose gel electrophoresis was performed on all amplicons to determine success of the amplification reaction and to identify differences in electrophoretic mobility that might suggest a gene insertion or deletion of more than 100 nucleotides. The amplification and sequencing strategies are described in Table S1 (supplemental material, online). A total of 74 single nucleotide polymorphisms (SNPs) were identified in these 23 individuals, 29 of which had a minor allele frequency ≥5% (Table S2, supplemental material, online). To determine whether the E292V mutation occurred on a common haplotype background, we computationally inferred *ABCA3* haplotypes using a Bayesian approach implemented in the PHASE computer software, and the 29 detected variants with minor allele frequency ≥5% (18).

We confirmed all mutations detected by restriction enzyme digestion or 5′ nuclease assay with direct sequencing as described in the supplemental material, online.

Data analysis. We used Phred, Phrap, PolyPhred, and Consed (http://www.phrap.org/phredphrapconsed.html) to identify and annotate SNPs in sequencing chromatograms and Prettybase (http://pga.mbt.washington.edu) to extract a final file with genotypes. We used SAS (Version 9.1.3, SAS Institute, Cary, NC) to perform χ^2 tests to determine distribution differences of categorical clinical characteristics and to perform Fisher's exact probability test to assess mutation frequency differences between groups. The Kruskal-Wallis test was used to compare ranked clinical characteristics where data normality and homoscedasticity were not assumed or Fischer's exact tests to determine differences in mutation frequency and categorical clinical characteristics and Kruskal-Wallis tests to compare numerical clinical characteristics (19). The SAS POWER procedure was also used to estimate statistical detection power separately in blacks (102 controls, 70 cases) and whites (77 controls, 142 cases) among our case–control cohort. In blacks, power was 36% when 5% dominant risk allele frequency difference or 13% recessive risk allele frequency difference was assumed, and power reached 88% when 11% dominant or 23% recessive risk allele frequencies were assumed. With the same assumptions, power estimates in whites were 43% and 93%, respectively.

To determine whether variants located near exons in *ABCA3* had the potential to affect RNA splicing, we used a splice site prediction application available through the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/seq_tools/splice.html).

Table 1. *Characteristics of case-control cohort*

	CON	RDS
Race		
Black	102	70
White	77	142
Other/missing	2	27
Sex		
Male	90	134
Female	91	102
Missing	0	3
Birth weight (kg)		
Mean ± SD	3.1 ± 0.5	2.0 ± 1.1
EGA (wks)		
Mean ± SD	39 ± 2	33 ± 5

RESULTS

Population-based cohorts. The 121ins2 mutation was not identified in the South African or Korean cohorts, but was similar in frequency in the Norwegian and Missouri cohorts (Table 2). Because we did not find I73T in over 4000 samples from the Missouri cohort, we decided not to screen other population-based cohorts for this mutation. We found E292V in 0.3–0.4% of the predominantly European descent cohorts (Norway and Missouri) but in <0.1% of the Asian or African descent cohorts (Table 2).

Case–control cohort. No infants in the RDS or CON groups carried the I73T mutation, and one infant in the RDS group carried the 121ins2 mutation (Table 3). In contrast, the prevalence of E292V in the RDS cohort was 10-fold higher than that in the Missouri cohort (3.8%, $p < 0.001$); the prevalence of E292V in the CON cohort was not different from that of the Missouri cohort (1.1%, $p = 0.2$). One set of twins in each of the CON and RDS groups was positive for E292V. No patient heterozygous for E292V in the RDS group carried SNPs that would be predicted to disrupt splice site junctions or alter ABCA3 protein sequence (Table 4), nor were any large insertions or deletions detected. Furthermore, with the exception of one synonymous SNP (S1372S), all potentially functional SNPs were identified in one or more of the 12 comparison individuals. Individual RDS8, who died after 8 mo of mechanical ventilation, was the only one for whom lung histology was available. There was variable, but extensive interstitial fibrosis and pneumocyte hyperplasia, along with

Table 2. *Population-based frequencies*

	Norway	South Africa-black	Korea	Missouri	Overall *p* value across cohorts
121ins2	3/2501 (0.1%)	0/2044 (0%)	0/2596 (0%)	8/10,044 (0.08%)*	0.2
I73T	—	—	—	0/4464 (0%)	—
E292V	8/2515 (0.3%)	0/1686 (0%)	0/1541 (0%)	4/1107 (0.4%)	0.004

* Reported in Ref. 17.

Table 3. *Disease-based frequencies*

	CON (N = 181)	RDS (N = 239)	*p* value* between groups
121ins2	0	1 (0.4%)	1.0
I73T	0	0	—
E292V	2 (1%)	9 (3.8%)	0.12

* Fisher's exact probabilities.

alveolar macrophages, but without alveolar proteinosis. Normal appearing lamellar bodies and tubular myelin were seen by electron microscopy (3,14,15,20).

Among the 15 *ABCA3* haplotypes computationally inferred from the E292V and control cohorts ($n = 23$), we identified two common haplotypes among the 35 alleles that did not carry E292V: haplotype 1 with 13 (37%) and haplotype 2 with 9 (26%) (Table 5). Among the 11 E292V carrying alleles, we identified one common haplotype ($n = 8$) and three unique haplotypes. The E292V haplotypes have two distinct blocks in common with each of the two most common nonE292V haplotypes: a 5–7 locus block from intron 1 to exon 9, seen in haplotype 2, and a 23–25 locus block from intron 8 through intron 28, seen in haplotype 1. This combined haplotype background (CCAAACCCGG. . .) was not seen in the absence of E292V, suggesting that E292V arose in conjunction with a recombination event between these two blocks and further suggests that the disease effect, if any, may not be solely because of E292V, but the unique combination of associated variants along the gene.

To assess whether E292V is associated with unique RDS characteristics, we compared demographic and clinical features in groups of RDS infants with and without E292V. Although the size of the cohort rendered limited statistical detection power, it appeared that the infants with E292V had a higher incidence of pneumothoraces (Table 6). The two most immature newborns with E292V (29 and 26 wk gestation) had evidence of more respiratory dysfunction (mechanical ventilation for >1 y and death because of chronic lung disease at 8 mo, respectively) than observed in newborn of similar gestational ages without E292V.

Table 4. *Exonic and splice site SNPs identified in subjects with E292V*

Subject	Race/Sex	SNP(s)*	Location	Number of non-E292V controls with variant	BW (kg)	GA (wks)	Outcome
CON1	B/M	Rs170447 (A)†	Ex 13 +30	1	3.6	41	
		Rs323043 [P585P] (B)†	Ex 14	7			
CON2	W/F	A, B			2.6	37	
RDS1	W/M	A, B			3.6	38	PTX; vent 4 d, O_2 5 d; discharged RA
RDS2A	W/F	A, B			2.3	35	Vent 10 d, O_2 5 d; discharged RA
RDS2B	W/F	B			2.1	35	PTX; vent 14 d, O_2 4 d; discharged RA
RDS3	W/M	16669 A	Ex 5 −11	2			Vent 1 y, O2 1 y
		Rs313908	Ex 18 −33	0			
		Rs313909	Ex 18 +34	0			
		Rs149532 [S1372]	Ex 26	0			
RDS4	W/M	B			2.9	37	PTX; vent 10 d, O_2 6 d; discharged RA
RDS5	W/M	45305	Ex 17 −17	1	3.8	36	Vent 4 d, O_2 1 d; discharged RA
RDS6	W/F	Rs149532 [S1372S]	Ex 26	0	2.5	33	Vent for 12 d, O2 for 18 d, then RA; died at 8 wks, non respiratory causes
RDS7	W/F	21289 [A227A]	Ex 7	1	3.1	39	CPAP for 2 d,
		Rs13332547	Ex 9 −20	3			O_2 for 4 d; discharged RA
		Rs13332514 [F353F]	Ex 9	3			
RDS8	W/M	None			0.7	26	Died at 8 mo, entirely vent dependent
RDS9	W/M	None			2.6	37	PTX, vent for 13 d, O_2 for 6 mo

* rs numbers through dbSNP (http://www.ncbi.nlm.nih.gov/SNP); if no rs number available, then the number refers to the genomic location from the ABCA3 sequence generated by Seattle SNPs (http://pga.gs.washington.edu/data/abca3/abca3.ColorFasta.html).

† A, B: common variants found in multiple individuals.

PTX, pneumothorax; Vent, mechanical ventilation; RA. room air.

Table 5. *Computational haplotypes identified in 23 individuals*

Group	Haplotype #	Haplotype	Alleles, N (%)*
nonE292V	1	CCGACCCCGGCAATCCGAAACTACAGGGTA	13 (37)
	1a	CCGACCCCGGCADCTCCAAAGCACTGGGCC	1 (3)
	1b	CCGACCCCGGCGDCTGCGGACCGCATAATC	1 (3)
	1c	CCGACCCCGGAGDTTCCGGACCGCATAATC	2 (6)
	1d	CCGACCCTGGCAATCCGAAACTACAGGGTA	1 (3)
	1e	CCGACTTTGGAGDTTCCGGACCGCATAATC	2 (6)
	1f	TTGACCCCGGCAATCCGAAACTACAGGGTA	1 (3)
	1g	TTGACTTTGGAGDTTCCGGACCGCATAATC	2 (6)
nonE292V	2	CCAAACCTAACGDCTGGAAGGCATTGGGCC	9 (26)
	2a	CCAAACCTAACADCTGGAAGGCATTGGGCC	2 (6)
	2b	CCAAACCTAACGDCTGGAAGCCATTGGGCC	1 (3)
E292V	3	CCAT†ACCCGGCAATCCGAAACTACAGGGTA	8 (73)
	3a	CCATACCCGGCAATCCGAAGCTACAGGGTA	1 (9)
	3b	CCATACCCGGCAATTCGAAACTACAGGGTC	1 (9)
	3c	CCATACCCGACAATCCGAAACTACAGGGTA	1 (9)

* Percent of alleles with or without E292V.

† E292V is an A>T transversion; shaded CC is indeterminate as to which haplotype from which it was derived.

Table 6. *Comparison of E292V positive newborns with RDS cohort (including twins)*

	E292V with RDS N = 10	RDS without E292V N = 237	*p* value
BW	2.6 (0.7–3.8)	1.8 (0.5–4.5) 201	0.10*
EGA	36 (26–39)	34 (23–43) 218	0.30*
Race (ED/AD)	10/0	139/72/26w	0.04†
Sex (F/M)	4/6	101/133	1.0†
Pneumothorax	4 (40%)	29 (15%)	0.06†
Duration mech vent/CPAP	8 (1–450)	14 (0–358) 171	0.46*
Duration O2	18 (3–800)	26 (0–358) 171	0.90*
Outcome at discharge			
On O2	3 (38%)	75 (43%)	0.71*
Vent	1 (10%)	9 (5%)	0.24*
Survive	8 (80%)	147 (86%)	0.64*

* Kruskal-Wallis test.

† Fisher's exact test.

DISCUSSION

The autosomal recessive 121ins2 (*SFTPB*) and autosomal dominant I73T (*SFTPC*) mutations most frequently detected in infants with lethal surfactant deficiency and interstitial lung disease are rare in the general population and in unselected infants with RDS, observations that suggest that these specific mutations have low population-attributable risk of RDS. *SFTPB* haploinsufficient murine lineages that demonstrate decreased lung compliance and air trapping at birth suggest that infants heterozygous for 121ins2 may have increased risk or severity of RDS (21,22). However, the observation that 121ins2 heterozygous siblings and parents of SP-B deficient infants are asymptomatic at birth coupled with the rarity of loss of function mutations in *SFTPB* suggests that these mutations would not be over-represented in a small cohort of premature and term infants with RDS (23,24). Although infants with mutations in *SFTPC* can present as RDS in the newborn period, most children with these mutations become symptomatic with interstitial lung disease beyond the newborn period, especially those with I73T (13,25). Therefore, it is also not surprising that we did not find I73T in the cohort of children who developed respiratory disease at birth.

We only screened for E292V in *ABCA3*, and, whereas the E292V carrier frequency is 3- to 5-fold higher than 121ins2 or I73T carrier frequencies in population-based cohorts of primarily European descent (Norway and Missouri) and we cannot exclude the possibility that other mutations in ABCA3 are just as common, the 10-fold enrichment in E292V prevalence in our RDS cohort suggests that E292V may increase the risk and/or severity of RDS in susceptible newborns. The developmental, environmental, and/or genetic background factors that contribute to differences in penetrance of these mutations remain to be defined.

Although disease-causing variants other than 121ins2 and E292V in *SFTPB* and *ABCA3* are highly prevalent in cohorts of infants selected for lethal respiratory distress, they have primarily been identified in single individuals or families (3,14,26). We did not have sufficient power to pursue the contribution of these more rare mutations to the disease-based risk for RDS in our neonatal intensive care unit cohort that was not enriched for lethal RDS. Common variants in *SFTPB* and *SFTPC* with minor allele frequencies ≥ 0.2 have also been associated with the risk for RDS, but the mechanisms by which these variants and combinations thereof impart risk are undetermined (27–30). To our knowledge, the contribution of common variants in *ABCA3* to the risk of RDS has not been evaluated. In view of the contributions of *SFTPB*, *SFTPC*, and *ABCA3* to surfactant function necessary for successful fetal-neonatal pulmonary transition, the low population-attributable risk of genetically disruptive mutations is not surprising.

The mechanism by which E292V may disrupt surfactant synthesis is unknown. Codon 292 is part of an intracellular loop between two transmembrane domains of the ABCA3 protein and the substitution of a nonpolar hydrophobic amino acid for a negatively charged hydrophilic amino acid may disrupt phospholipid binding and transport into the lamellar body. *In vitro* studies suggest a primary role for *ABCA3* mutations in the disruption of pulmonary surfactant metabolism through abnormalities in intracellular protein trafficking,

defects in ATP hydrolysis, or abnormal phospholipid packaging (3–6,31,32). In addition, ABCA3 protein expression has been shown to increase with advancing gestation (33). These observations, along with other reports of lethal and chronic respiratory disease in the presence of a single mutation in *ABCA3*, suggest that E292V itself, or through interactions with variants in other genes, could disrupt ABCA3 function in developmentally susceptible individuals (14,16,26,34). In contrast to those reports, however, all but one infant in this study recovered from their lung disease.

The unique haplotype associated with E292V raises the possibility that a specific combination of variants within this new block could confer the phenotype. Our resequencing strategy that focused on coding regions and flanking sequence did not identify any coding or splice site variants either upstream or downstream of E292V that would obviously alter ABCA3 function, but it is possible that functional intronic or promoter variants reside within the regions that were not sequenced or in other genes along the surfactant synthetic pathway.

Taken together, these data suggest that E292V may increase RDS susceptibility in the context of currently unknown developmental, genetic, or environmental traits. Even though the 121ins2, I73T, and E292V mutations are rare in the general population, they are sufficiently prevalent to warrant evaluation in the context of respiratory compromise that seems disproportionate for gestational age.

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