Synonymous *ABCA3* Variants Do Not Increase Risk for Neonatal Respiratory Distress Syndrome

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Objective To determine whether synonymous variants in the adenosine triphosphate-binding cassette A3 transporter (*ABCA3*) gene increase the risk for neonatal respiratory distress syndrome (RDS) in term and late preterm infants of European and African descent.

Study design Using next-generation pooled sequencing of race-stratified DNA samples from infants of European and African descent at ≥34 weeks gestation with and without RDS (n = 503), we scanned all exons of *ABCA3*, validated each synonymous variant with an independent genotyping platform, and evaluated race-stratified disease risk associated with common synonymous variants and collapsed frequencies of rare synonymous variants.

Results The synonymous *ABCA3* variant frequency spectrum differs between infants of European descent and those of African descent. Using in silico prediction programs and statistical strategies, we found no potentially disruptive synonymous *ABCA3* variants or evidence of selection pressure. Individual common synonymous variants and collapsed frequencies of rare synonymous variants did not increase disease risk in term and late-preterm infants of European or African descent.

Conclusion In contrast to rare, nonsynonymous *ABCA3* mutations, synonymous *ABCA3* variants do not increase the risk for neonatal RDS among term and late-preterm infants of European or African descent. *(J Pediatr 2014;164:1316-21)*.

Neonatal respiratory distress syndrome (RDS) results from insufficiency of pulmonary surfactant, a phospholipid–protein complex that is synthesized, packaged, and exocytosed by alveolar type 2 cells, decreases surface tension, and maintains alveolar expansion at end expiration.[1] RDS is generally attributed to developmental insufficiency of pulmonary surfactant production; however, genetic mechanisms also contribute to the risk for neonatal RDS.[2-7]

Adenosine triphosphate-binding cassette A3 transporter (ABCA3) is a member of the highly conserved family of adenosine triphosphate binding cassette transporters that bind and hydrolyze adenosine triphosphate to transport substrates across cellular membranes.[8] ABCA3 is most highly expressed in the lung and is localized to the limiting membranes of lamellar bodies, intracellular storage organelles of pulmonary surfactant.[9,10] Rare, recessive, nonsynonymous mutations in *ABCA3* are associated with lethal neonatal RDS and chronic respiratory disease in children.[5,11] Recently, single, rare, nonsynonymous mutations in *ABCA3* were associated with reversible RDS in term and late-preterm infants of European descent.[7]

Although nonsynonymous *ABCA3* mutations that change the amino acids coded into that protein are known to increase the risk of neonatal RDS,[5,7,12] much less is known about synonymous variants that do not change the amino acid sequence but may alter intron–exon splicing, splicing control elements, messenger RNA stability, translation efficiency, or protein folding.[13-18] Two synonymous *ABCA3* variants have been associated with the risk of neonatal RDS.[19,20] The synonymous variant p.F353F, which resides in the transmembrane domain, was associated with a prolonged course of RDS in preterm Finnish infants,[19] and p.P585P, which resides in the nucleotide binding domain, was overrepresented in preterm Chinese infants with RDS.[20]

Given that mutations in *ABCA3* can cause severe neonatal RDS, the evaluation of term and late-preterm infants with progressive respiratory failure unresponsive to medical management frequently includes *ABCA3* sequencing to establish a diagnosis of ABCA3 deficiency.[21] Because most *ABCA3* mutations are rare, private, and have not been evaluated in surrogate cell systems,[22,23] clinicians must rely on results of in silico prediction algorithms[24-26] and the opinions of experts. Even though synonymous variants are frequently identified with such genetic sequencing, prognostic information for these variants is limited. Thus, using

ABCA3	Adenosine triphosphate-binding cassette A3 transporter
ESP	Exome Sequencing Project
MAF	Minor allele frequency
RDS	Respiratory distress syndrome

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high-resolution, high-throughput, next-generation exonic sequencing; computational algorithms for variant discovery; in silico programs to predict functionality; independent validation of variants; and statistical strategies to compare common synonymous variant and collapsed rare synonymous variant frequencies, we examined the associations of synonymous *ABCA3* variants with the risk of neonatal RDS in term and late-preterm infants of European and African descent.

Methods

We used DNA collected from a previously reported prospectively enrolled cohort of newborn infants with and without RDS, ≥34 weeks gestational age, and maternally designated European or African descent recruited from the nurseries at Washington University Medical Center[7] (**Table I**). We defined RDS as a requirement for supplemental oxygen (fraction of inspired oxygen ≥0.3), chest radiograph findings consistent with RDS, and the need for continuous positive airway pressure or mechanical ventilation within the first 48 hours of life.[6,7] Infants without RDS (non-RDS group) had no respiratory symptoms and were hospitalized for other neonatal problems. We assigned gestational age based on the best obstetrical estimate, and we excluded infants with cardiopulmonary malformations, pulmonary hypoplasia, culture-positive sepsis, chromosomal anomalies, known surfactant mutations, or rapidly resolving RDS (within <24 hours of birth). We randomly excluded 1 of each set of monozygotic twins (n = 3) and twins in whom zygosity could not be reliably determined (n = 2). We extracted details of the respiratory course and outcome from the clinical chart. This study was reviewed and approved by the Washington University School of Medicine's Human Research Protection Office.

DNA Isolation and Pool Preparation

We isolated DNA from blood samples using Puregene DNA isolation kits (Qiagen, Valencia, California)[6,7] and combined equimolar amounts from each individual into 4 race-stratified pools: infants of African descent with RDS (n = 44), infants of African descent without RDS (n = 196), infants of European descent with RDS (n = 112), and infants of European descent without RDS (n = 161).

Next-Generation Sequencing and Validation

We used an Illumina next-generation sequencing platform to sequence all exons and flanking regions (approximately 50 base pairs) of *ABCA3* (data available on request).[27] To optimize the selection of significance thresholds for detection of rare variants in each sequencing run, we added a 1934-bp oligonucleotide without variation and a 335-bp oligonucleotide containing 15 known insertions, deletions, and substitutions at a frequency of <1 allele per pool.[28] Inclusion of negative and positive controls allowed run-specific error models to achieve high sensitivity (0.99) and specificity (0.99) for detecting rare variants within each pool. We sequenced approximately 37 kb per individual, with a mean coverage of 82×.

We then used the computational algorithm SPLINTER[28] to detect rare (ie, minor allele frequency [MAF] <0.01) and common (MAF ≥0.01) synonymous variants. Each variant was confirmed with an independent genotyping strategy (Sequenom, TaqMan, or Sanger resequencing) and linked to its individual sample (data available on request). We had insufficient DNA for 10 infants (4 of African descent and 6 of European descent) to complete the validation studies, and thus we excluded these infants from all further analyses (**Table I**).

In Silico Prediction of Functionality

We used Alamut 2.3 (Interactive Biosoftware, Rouen, France), which combines the results of 7 splicing prediction algorithms— Human Splicing Finder (www.umd.be/HSF),[29] GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer),[30] MaxEntScan (genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html),[31] NNSplice,[32] Splice Site Finder-Like,[33] ESE-Finder (http://rulai.cshl.edu/tools/ESE), and RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/)—to predict whether a synonymous variant would alter predicted intron–exon splicing patterns. We used the Genome Variation Server (http://gvs.gs.washington.edu/GVS137/index.jsp) to determine whether the common (MAF ≥0.01) synonymous variants were in linkage disequilibrium.

Statistical Analyses

We used χ^2 and Fisher exact tests to determine whether common *ABCA3* synonymous variants (MAF ≥0.01) were in Hardy-Weinberg equilibrium. We compared race-stratified frequencies of individual common (MAF ≥0.01) synonymous

Table I. Characteristics of European and African descent disease-based groups (n = 503)

	European descent			African descent		
	RDS (n = 109)	Non-RDS (n = 158)	*P*	RDS (n = 44)	Non-RDS (n = 192)	*P*
Sex, n (%)						
Female	45 (41)	72 (46)	.49	10 (0.23)	99 (0.52)	<.001
Male	64 (59)	86 (54)		34 (0.77)	93 (0.48)	
Gestational age, wk, mean ± SD	36.9 ± 1.7	38.2 ± 1.6	<.001	37.6 ± 2.7	38.9 ± 1.7	.003
Birth weight, kg, mean ± SD	3.1 ± 0.6	3.1 ± 0.7	.50	2.9 ± 1.0	3.1 ± 0.4	.07
Route of delivery, n (%)						
Vaginal	54 (0.49)	71 (0.45)	.53	15 (0.34)	131 (0.68)	<.001
Cesarean	55 (0.51)	87 (0.55)		29 (0.66)	61 (0.32)	