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Author manuscript
Pediatr Pulmonol. Author manuscript; available in PMC 2022 May 29.

Published in final edited form as:
Pediatr Pulmonol. 2022 May ; 57(5): 1325–1330. doi:10.1002/ppul.25862.

Biologic characterization of ABCA3 variants in lung tissue from infants and children with ABCA3 deficiency

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Abstract

ABCA3 is a phospholipid transporter protein required for surfactant assembly in lamellar bodies of alveolar type II cells. Biallelic pathogenic *ABCA3* variants cause severe neonatal respiratory distress syndrome or childhood interstitial lung disease. However, *ABCA3* genotype alone does not explain the diversity in disease presentation, severity, and progression. Additionally, monoallelic *ABCA3* variants have been reported in infants and children with ABCA3-deficient phenotypes. The effects of most *ABCA3* variants identified in patients have not been characterized at the RNA level. *ABCA3* allele-specific expression occurs in some cell types due to epigenetic regulation. We obtained lung tissue at transplant or autopsy from 16 infants and children with ABCA3 deficiency due to compound heterozygous *ABCA3* variants for biologic characterization of the predicted effects of *ABCA3* variants at the RNA level and determination of *ABCA3* allele expression. We extracted DNA and RNA from frozen lung tissue and reverse-transcribed cDNA

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

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from mRNA. We performed Sanger sequencing to assess allele-specific expression by comparing the heights of variant nucleotide peaks in amplicons from genomic DNA and cDNA. We found similar genomic and cDNA variant nucleotide peak heights and no evidence of allele-specific expression among explant or autopsy samples with biallelic missense *ABCA3* variants (*n* = 6). We observed allele-specific expression of missense alleles in *trans* with frameshift (*n* = 4) or nonsense (*n* = 1) variants, attributable to nonsense-mediated decay. The missense variant c.53 A > G;p.Gln18Arg, located near an exon-intron junction, encoded abnormal splicing with skipping of exon 4. Biologic characterization of *ABCA3* variants can inform discovery of variant-specific disease mechanisms.

Keywords

ATP-binding cassette transporter A3; childhood interstitial lung disease (chILD); neonatal respiratory distress syndrome (RDS); pediatric lung transplant; surfactant metabolism dysfunction

1 | INTRODUCTION

ATP-binding cassette member A3 (ABCA3) is a highly conserved, transmembrane spanning protein that localizes to the limiting membrane of lamellar bodies in alveolar epithelial type II cells, transports phospholipids, and is required for pulmonary surfactant assembly.[1-4] Biallelic variants in *ABCA3* are associated with diverse lung phenotypes in newborn infants, children, and adults.[5-9] Infants with biallelic loss-of-function *ABCA3* variants (e.g., nonsense and frameshift) present with severe neonatal respiratory distress syndrome (NRDS) and die within the first year of life without lung transplantation.[5,7] However, the disease presentation, progression, and severity for infants and children with other types of *ABCA3* variants including missense, in-frame insertions/deletions, or intronic variants that alter RNA splicing are more difficult to predict.[5,7] In addition, reports of infants and children with monoallelic *ABCA3* variants and ABCA3-deficient phenotypes (NRDS, childhood interstitial lung disease [chILD])[1,10-13] suggest that allele-specific expression may contribute to phenotypic diversity.[14,15]

For most of the *ABCA3* variants identified in patients with ABCA3 deficiency, there is no biological characterization of their effect at either the RNA or protein level. Studying the effects of *ABCA3* variants on transcript levels of each allele could identify abnormal splicing, reduced transcription, or RNA degradation. In addition, a potential mechanism to account for phenotypic diversity among individuals with biallelic or monoallelic *ABCA3* variants may be allele-specific expression, as suggested by Savova and colleagues.[16] Allele-specific expression of human autosomal genes contributes to somatic cellular mosaicism, differences in gene expression patterns in specific cell types, and balancing selection.[16-19]

To perform biologic validation of *ABCA3* variants and to determine if *ABCA3* alleles are equally expressed in the human lung, we characterized *ABCA3* allele expression in lung tissue from infants and children with compound heterozygous *ABCA3* variants who underwent bilateral lung transplantation or died from respiratory failure.

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2 | MATERIALS AND METHODS

2.1 | Lung explant selection

We obtained lung tissue snap frozen at the time of bilateral lung transplantation for progressive respiratory failure from 14 infants and children with ABCA3 deficiency due to compound heterozygous *ABCA3* variants at St. Louis Children's Hospital from 1999 to 2019. We obtained frozen lung tissue samples from the autopsies of two infants with ABCA3 deficiency who died of respiratory failure before 4 months of age (Subjects 9 and 10). Genotype information for 13 subjects was previously reported.[5] This study was approved by the Washington University Human Research Protection Office, and parents provided informed consent for participation.

2.2 | Genomic analysis

We extracted genomic DNA and RNA from snap-frozen lung tissue using AllPrep DNA/RNA Mini Kit® (Qiagen, Germantown, MD) from both left and right lungs ($n = 11$) or from two different lung tissue samples ($n = 5$) according to the manufacturer's instructions. We reverse-transcribed cDNA from total RNA using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). We designed PCR primers that produced genomic DNA or cDNA amplicons which included the *ABCA3* variants for each subject and performed Sanger sequencing with an ABI 3130XL (Applied Biosystems). For the cDNA studies, we confirmed that the amplicons spanned at least one exon-exon junction. To evaluate transcript splicing, we purified specific cDNA amplicons after agarose gel electrophoresis using QIAquick Gel Extraction Kit® (Qiagen and Sanger sequencing. Genomic and cDNA primers are available in Table S1.

2.3 | Bioinformatic analysis

We analyzed sequences from genomic DNA using FinchTV (https://finchtv.software.informer.com/1.4/) to confirm the previously identified *ABCA3* variants. We identified differences in nucleotide peak intensity heights between genomic and cDNA sequences to determine allele-specific expression. To investigate possible differences in regional lung expression of *ABCA3*, we compared results from two lung tissue samples for each subject.

3 | RESULTS

We confirmed all previously identified *ABCA3* variants (Table 1). We observed similar relative transcript abundance among subjects who were compound heterozygous for *ABCA3* missense variants ($n = 6$) (Figures 1A, S1A-L). Among subjects with missense alleles in *trans* with frameshift ($n = 4$) or nonsense ($n = 1$) variants, we found differences in cDNA and genomic DNA nucleotide intensity heights due to nonsense mediated decay (NMD) of the mRNA transcript encoded by the frameshift or premature stop codons (Figure 1B). We identified transcript abundance as predicted by genotype for samples 4, 5, 11, 12 (Figures S1M-R). We found no differences in relative transcript abundance between left and right lung tissue samples ($n = 11$) or from different lung tissue samples ($n = 5$). Parental samples were available for 11 subjects. Five subjects (Subjects 1, 2, 3, 4, and 13) had 3 rare

ABCA3 variants (2 variants in cis) based on analysis of parental DNA (*n* = 3, Subjects 1, 4, and 13) or inferered based on nucleotide peak intensity heights (*n* = 2, Subjects 2 and 3). As expected, relative transcript abundance of variants in *cis* were similar. Consistent with previous genotype-phenotype correlation studies,[5,7] Subject 5 with biallelic frameshift variants and predicted NMD presented with NRDS, while infants and children with biallelic missense variants presented with either NRDS or chILD phenotypes.

One sample from a subject with chILD who carried 3 rare *ABCA3* missense variants (Subject 13, maternal allele: c.53 A > G;p.Gln18Arg and paternal allele:c.127 C > T;p.Arg43Cys/c.4958 C > T;p.Pro1653Leu) initially appeared to demonstrate greater relative abundance (i.e., allele-specific expression) of the transcript from the paternal allele. To evaluate relative transcript abundance in this lung explant, we first used primers that spanned exons 4 and 5 which contained both the c.53 A > G;p.Gln18Arg and c.127 C > T;p.Arg43Cys variants. When we compared nucleotide peak heights for the maternally inherited c.53 A > G;p.Gln18Arg variant in the genomic and cDNA, we observed apparent allele-specific expression of the paternal, wild-type allele (A) in cDNA as compared to genomic DNA (overlapping peak heights of A and G alleles (Figure S2). We also observed increased transcript abundance of the paternal c.127 C > T;p.Arg43Cys transcript in the cDNA, but the c.4958 C > T;p.Pro1653Leu variant from the same paternal allele demonstrated similar genomic and cDNA peak heights. To evaluate for potential aberrant splicing, we designed primers that targeted each of the 3 *ABCA3* variants identified in Subject 13. Specifically, we used primers that began in exon 3 and ended in exon 5 to amplify the region with the c.53 A > G;p.Gln18Arg variant and primers that began in exon 4 and ended in exon 6 to amplify the region with the c.127 C > T;p.Arg43Cys variant. We observed 2 cDNA products from primers which spanned exons 3 to 5, one migrating at the expected mobility (~350 base pairs [bp]) and one ~50bp smaller in length (Figure 2A). Using gel purification and Sanger sequencing, we confirmed a 54 bp deletion that corresponds to deletion of exon 4 in the smaller cDNA product (Figure 2B). Our initial finding of increased relative abundance of the paternal transcript (c.127 C > T;p.Arg43Cys) resulted from the position of the original PCR primer in the deleted exon 4 which prevented amplification of the region that included the c.53 A > G;p.Gln18Arg variant. The c.53 A > G;p.Gln18Arg missense variant, which is 2 bp from the exon 4-intron 4 junctions, alters transcript splicing, results in deletion of exon 4, and likely encodes a dysfunctional ABCA3 protein.

4 | DISCUSSION

ABCA3 deficiency results from biallelic, pathogenic *ABCA3* variants and causes diverse pulmonary phenotypes that range from severe neonatal respiratory failure that is lethal without lung transplant to chronic interstitial lung disease among infants, children, and adults.[1,5,7,8] A potential mechanism to account for phenotypic variability among individuals with biallelic or monoallelic *ABCA3* variants may be allele-specific expression, as suggested by Savova and colleagues (Supplement 4 Table in Reference 16). However, our results from lung tissue of infants and children with biallelic *ABCA3* missense variants demonstrated similar relative transcript abundance. It is possible that some *ABCA3* variants may cause these effects, but they were not sampled in this cohort. Epigenetic mechanisms

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including DNA methylation and histone modification result in the common endpoint of reduced transcript abundance that would be detectable by peak height comparison. However, our methods did not directly investigate modifications to DNA or chromatin. Our methods would not detect reduction of translation that may result from other epigenetic mechanisms including microRNAs (miRNAs) and short interfering RNAs (siRNAs). Furthermore, it is unknown whether the post-transcriptional effects of miRNAs and siRNAs would be allele-specific. We used frozen lung tissue samples which contained several types of pulmonary cells (e.g., alveolar epithelial type I and type II cells, club cells) to extract genomic DNA and RNA and assess allele-specific expression. Single-cell transcriptome analyses may reveal differences in allele expression in specific pulmonary cells such as alveolar type II cells that would be missed with our sampling strategy.

ABCA3 missense variants may disrupt intracellular trafficking of ABCA3 with retention in the endoplasmic reticulum (ER) or impair ATP-hydrolysis and phospholipid transport into the lamellar body.[20-24] Pulmonary phenotype diversity may be related to the amount of residual ABCA3 protein function, cellular mechanisms triggered by pathogenic variants (e.g., activation of ER stress pathways or autophagy),[5,7] variants in other surfactant genes (e.g., the surfactant protein C gene, *SFTPC*),[25] environmental factors including tobacco exposure[8,9] or other genetic modifiers. Siblings with the same *ABCA3* genotype are generally concordant for phenotype, although phenotypic discordance has been reported.[5,7,26] The most common *ABCA3* missense variant p.Glu292Val (gnomAD minor allele frequency 0.00234, gnomad.broadinstitute.org; accessed December 2021) is more frequently associated with chILD[5] than severe neonatal respiratory failure.

We found that the bioinformatic prediction of a missense amino acid change resulting from the c.53 A > G variant was incorrect. Rather, the c.53 A > G variant, located near an exon-intron junction, altered transcript splicing and resulted in skipping of exon 4. Variants near exon-intron junctions may encode loss of ABCA3 function by exon skipping or intron retention. A similar mechanism has been reported for the p.Glu145His missense variant (more accurately noted as p.Leu109_Gln145del) in *SFTPC*.[27] This *SFTPC* variant is located in the last base of exon 4 and results in abnormal splicing with skipping of exon 4 and a truncated protein with abnormal intracellular trafficking.[27] Our results also emphasize the importance of parental DNA samples to phase *ABCA3* variants as 5 (~30%) subjects had 3 *ABCA3* variants identified consistent with prior studies.[5,28]

Transcriptomic and genomic studies of snap frozen lung tissue at autopsy, lung transplant, or lung biopsy from patients with ABCA3 deficiency can advance understanding of *ABCA3* variant-specific mechanisms that disrupt pulmonary function. Although multiple cell types express *ABCA3* (gtexportal.org; accessed December 2021), lung tissue is required due to insufficient expression of *ABCA3* in blood, skin, and other accessible tissues. Thus, most variants identified in patients with ABCA3 deficiency have not been biologically characterized to determine their effects at the RNA or protein level, and bioinformatic predictions of variant effects may be incorrect. Understanding *ABCA3* variant-specific mechanisms of disruption of surfactant metabolism and lung function may inform pharmacologic therapies for patients with ABCA3 deficiency.[24,29,30] We recommend

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flash frozen lung tissue be obtained at time of biopsy, explant, or autopsy for patients with confirmed or suspected diagnoses of surfactant dysfunction disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to thank Larry Nogee, M.D., for his collaboration, thoughtful review, and advice. This study was supported by grants from the National Institutes of Health R01 HL149853 (Jennifer A. Wambach), R33 HL120760 (F. Sessions Cole), U01 HL134745 (F. Sessions Cole and Jennifer A. Wambach), and the Children's Discovery Institute (F. Sessions Cole and Jennifer A. Wambach).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study. These data were presented as a virtual poster presentation at the 2021 American Thoracic Society meeting (Kathryn K. Xu).

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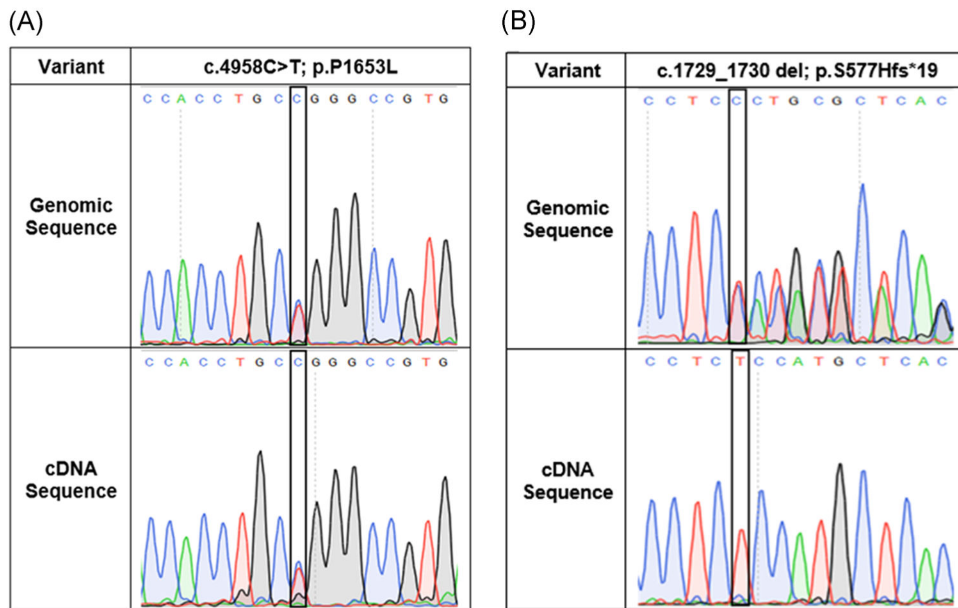

FIGURE 1.

(A) Genomic and cDNA sequencing of the c.4958 C > T;p.P1653L variant in Subject 13 demonstrates similar, overlapping wild-type allele (C, blue) and variant allele (T, red) peak heights (shown in black boxes) for genomic DNA and cDNA. (B) Genomic and cDNA sequencing of the c.1729_1730del;p.S577Hfs*19 frameshift variant in Subject 5 demonstrates increased transcript abundance of the wild-type allele (T) in cDNA compared to genomic DNA (overlapping peak heights of T and C alleles) (boxes) due to nonsense mediated decay of the mRNA transcript encoded by the frameshift allele

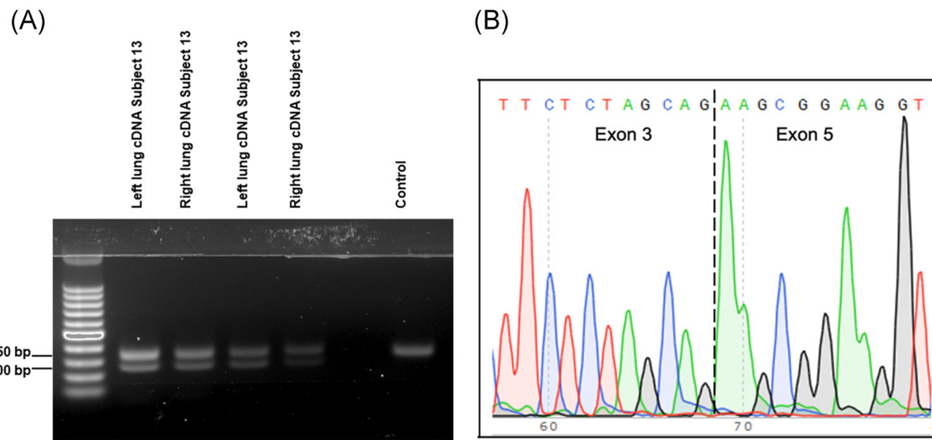

FIGURE 2.

(A) Agarose gel of cDNA products using primers that targeted exons 3 to 5 from Subject 13 compared to a control NRDS infant without *ABCA3* variants. (B) Sanger sequencing revealed that the upper band (~350 bp) corresponds to the wild-type transcript and the lower band (~300 bp) includes a 54 bp deletion which corresponds to deletion of exon 4

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TABLE 1

Subjects with ABCA3 deficiency due to biallelic ABCA3 variants

Subject	Phenotype	Age at transplant or death	Allele 1	Allele 2
1	NRDS	4 months	c.128 G > A; p.Arg43His/ c.4747 C > T; p.Arg1583Trp	c.1858_1862 del; p.Asn620Aspfs*22
2	NRDS	4 months	c.537 G > C; p.Trp179Cys/ c.2309 C > T; p.Pro770Leu	c.1382_1383 del; p.Val461Alafs*48
3	NRDS	3 months	c.2296 C > T; p.Pro766Ser/c.2880 G > C; p.Leu960Phe	c.1729_1730 del; p.Ser577Hisfs*19
4	NRDS	10 months	c.838 C > T; p.Arg280Cys/ c.4765 C > T; p.Gln1589*	c.4195 G > A; p.Val1399Met
5	NRDS	4 months	c.817_821 del; p.Tyr273Argfs*138	c.1729_1730 del; p.Ser577Hisfs*19
6	NRDS	2 months	c.628 G > A; p.Gly210Ser	c.4675 C > T; p. Arg1559*
7	NRDS	4 months	c.629 C > T; p.Gly210Val	c.817_821 del; p.Tyr273Argfs*138
8	NRDS	3 months	c.757 G > T; p.Asp253Tyr	c.4784 T > C; p.Leu1595Pro
9	NRDS	3 months	c.1240 G > A; p.Ala414Thr	c.4376 G > A; p.Gly1459Asp
10	NRDS	2 months	c.2945 T > C; p.Leu982Pro	c. 3661 G > A; p.Gly1221Ser
11	NRDS	2 months	c.3 G > C; p.Met1?	c.607_609del; p.Glu203del
12	NRDS	5 months	c.1112–20 G > A; splice site	c.3163_3172delGCCGTCGTGG; p.Ala1055Thrfs*76
13	chILD	2 years	c.127 C > T; p.Arg43Cys/ c.4958 C > T; p.Pro1653Leu	c.53 A > G; p.Gln18Arg
14	chILD	12 years	c.875 A > T; p.Glu292Val	c.3341 C > T; p.Thr1114Met
15	chILD	5 years	c.622 C > T; p.Arg208Trp	s.2279 T > G; p.Met760Arg
16	chILD	2 years	c.1240 G > A; p.Ala414Thr	c.4271 C > T; p.Thr1424Met

*Genotype data for subjects 1–4, 6, 8–15 were previously reported.[5]