

ORIGINAL ARTICLE

ABCA3 Gene Mutations in Newborns with Fatal Surfactant Deficiency

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ABSTRACT

BACKGROUND

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Pulmonary surfactant forms a lipid-rich monolayer that coats the airways of the lung and is essential for proper inflation and function of the lung. Surfactant is produced by alveolar type II cells, stored intracellularly in organelles known as lamellar bodies, and secreted by exocytosis. The gene for ATP-binding cassette transporter A3 (ABCA3) is expressed in alveolar type II cells, and the protein is localized to lamellar bodies, suggesting that it has an important role in surfactant metabolism.

METHODS

We sequenced each of the coding exons of the *ABCA3* gene in blood DNA from 21 racially and ethnically diverse infants with severe neonatal surfactant deficiency for which the etiologic process was unknown. Lung tissue from four patients was examined by high-resolution light and electron microscopy.

RESULTS

Nonsense and frameshift mutations, as well as mutations in highly conserved residues and in splice sites of the *ABCA3* gene were identified in 16 of the 21 patients (76 percent). In five consanguineous families with mutations, each pair of siblings was homozygous for the same mutation and each mutation was found in only one family. Markedly abnormal lamellar bodies were observed by ultrastructural examination of lung tissue from four patients with different *ABCA3* mutations, including nonsense, splice-site, and missense mutations.

CONCLUSIONS

Mutation of the *ABCA3* gene causes fatal surfactant deficiency in newborns. *ABCA3* is critical for the proper formation of lamellar bodies and surfactant function and may also be important for lung function in other pulmonary diseases. Since it is closely related to *ABCA1* and *ABCA4*, proteins that transport phospholipids in macrophages and photoreceptor cells, it may have a role in surfactant phospholipid metabolism.

N Engl J Med 2004;350:1296-303.

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PULMONARY SURFACTANT IS A COMPLEX mixture of lipids and proteins that is essential for normal lung function. Surfactant lowers surface tension at the air-liquid interface, thereby preventing end-expiratory atelectasis. It is stored within alveolar type II cells in organelles containing multiple phospholipid layers, known as lamellar bodies, and is secreted into the alveoli by exocytosis. The production of pulmonary surfactant is developmentally regulated, and the respiratory distress syndrome may develop in premature infants owing to the lack of surfactant. Homozygous loss-of-function mutations in the gene encoding the hydrophobic surfactant protein B (*SFTPB*) results in fatal surfactant deficiency in full-term newborns.¹ Fatal respiratory disease has been reported in full-term infants with symptoms of surfactant deficiency in whom a deficiency of surfactant protein B was excluded, and the occurrence of familial cases suggests that there are additional genetic mechanisms.²

The genes for ATP-binding cassette (ABC) transporters encode membrane proteins involved in the transport of compounds across biologic membranes, and 14 ABC genes have been associated with distinct genetic diseases in humans.³ Several ABC transporters are involved in the transport of phospholipids and sterols. The gene encoding ABC transporter 1 (*ABCA1*) is mutated in Tangier disease, a disorder involving the accumulation of cholesterol in macrophages and peripheral tissues and a deficiency of high-density lipoproteins.⁴⁻⁶ The *ABCA4* gene is expressed in photoreceptors and encodes a protein that has been implicated in transporting retinal-phosphatidylethanolamine complexes in the photoreceptor membrane disks of rods.^{7,8} The *ABCA4* gene is mutated in several recessive disorders that involve retinal degeneration, including macular dystrophy due to Stargardt's disease, most recessive forms of cone-rod dystrophy, and some recessive forms of retinitis pigmentosa.^{9,10} The *ABCG5* and *ABCG8* genes are expressed in the liver and intestine and are mutated in patients with sitosterolemia, a disorder involving the accumulation of cholesterol and other sterols.^{11,12}

The *ABCA3* gene encodes a 1704-amino-acid protein highly expressed in the lung, which has been localized to the limiting membrane of lamellar bodies,^{13,14} implicating *ABCA3* as possibly important in the maturation of lamellar bodies and surfactant production. Because of the probable role of *ABCA3* in lipid transport, its location within

alveolar type II cells, and the association of other ABC genes with human diseases, we conducted a study to determine whether *ABCA3* is involved in surfactant phospholipid metabolism and whether *ABCA3* is a candidate gene for unexplained surfactant deficiency in full-term infants.

METHODS

PATIENTS

From July 1995 until April 2003, blood samples were collected from 337 infants with severe respiratory disease as part of a study to identify inherited abnormalities of surfactant metabolism. The infants were of northern and southern European, African American, Asian, and Middle Eastern origin. All infants were born after a gestation of at least 36 weeks and had persistent hypoxemic respiratory failure, with no known cause for their respiratory disease identified at the time of enrollment. The onset of respiratory symptoms had occurred within hours after birth, and all infants had clinical or radiographic findings (or both) that were consistent with surfactant deficiency.

A cause of the lung disease was subsequently identified in 15 infants: alveolar capillary dysplasia in 4, total anomalous pulmonary venous return in 4, viral pneumonia in 3, acinar dysplasia in 2, pulmonary lymphangiectasia in 1, and mucopolysaccharidosis type II in 1. In 47 infants (14 percent), hereditary deficiency of surfactant protein B was identified as the basis of the lung disease, as determined by the identification of loss-of-function mutations on both alleles of the *SFTPB* gene. Deficiency of surfactant protein B was ruled out in the remaining 275 infants by a combination of protein analyses of lung fluid and tissue and genetic studies.^{15,16} Among these infants, 121 were analyzed for mutations in the gene for surfactant protein C (*SFTPC*); 6 of these infants were found to carry such mutations.

Of the remaining 115 infants, a subgroup of 21 infants from 14 families who were likely to have a genetic basis for their lung disease, on the basis of a family history of a similarly affected sibling, consanguinity, or both, or who had fatal disease in association with low surfactant protein levels in tracheal-aspirate fluid, was selected for analysis of the *ABCA3* gene. These infants included six pairs of siblings, one of which was known to have abnormal lamellar bodies.¹⁷ The majority of these infants died within a month after birth (Table 1).

Table 1. Characteristics of Full-Term Infants with Clinical Surfactant Deficiency.*

Patient No.	Race or Ethnic Group	Sex	Family No.	Family History/Consanguinity	Outcome	Histologic Findings	ABCA3 Mutation
1	White	F	1	Yes/Yes	Death within 3 mo after birth	DIP, PAP	W1142X/W1142X
2	White	F	1	Yes/Yes	Death during neonatal period	DIP, PAP	W1142X/W1142X
3	Black	M	2	Yes/Yes	Death during neonatal period	NA	L101P/L101P
4	Black	M	2	Yes/Yes	Death during neonatal period	NA	L101P/L101P
5	White	F	3	Yes/No	Death during neonatal period	NA	4552insT/L1580P
6	White	F	3	Yes/No	Death during neonatal period	NA	4552insT/L1580P
7	White	M	4	Yes/No	Death within 3 mo after birth	PAP	G1221S/L982P
8	White	M	4	Yes/No	Death during neonatal period	PAP	G1221S/L982P
9	Middle Eastern	M	5	Yes/Yes	Death during neonatal period	DIP, PAP	L1553P/L1553P
10	Middle Eastern	M	5	Yes/Yes	Death during neonatal period	NA	L1553P/L1553P
11	White	M	6	Yes/No	Recovery from RDS	NA	None found
12	White	M	6	Yes/No	Recovery from RDS	NA	None found
13	Middle Eastern	M	7	No/Yes	Unknown	NA	1644delC/1644delC
14	Middle Eastern	M	8	Yes/No	Death during neonatal period	DIP, PAP	R106X/R106X
15	Asian	F	9†	Yes/Yes	Death during neonatal period	NA	4909+1G>A/4909+1G>A
16	White	M	10	Yes/Yes	Death during neonatal period	NA	None found
17	White	M	11	No/No	Recovery from RDS	NA	None found
18	White	F	12	No/No	Death during neonatal period	NA	None found
19	White	M	13	Yes/No	Chronic lung disease	CPI, DIP	Q1591P/—‡
20	Hispanic	M	14	No/No	Death after lung transplantation	PAP	N568D/—‡
21	Asian	F	9†	Yes/Yes	Death during neonatal period	PAP	4909+1G>A/4909+1G>A

* DIP denotes desquamative interstitial pneumonitis, PAP pulmonary alveolar proteinosis, NA not available, RDS respiratory distress syndrome, and CPI chronic pneumonitis of infancy. Race or ethnic group was self-assigned by the parents.

† Patients 15 and 21 were cousins.

‡ No mutation was identified on one allele.

DNA was prepared from whole blood from the infants with the use of a commercially available kit (Genra Systems). The protocol was approved by the institutional review boards of the participating institutions, and written informed consent was obtained from the parents for genetic studies.

DETECTION OF MUTATIONS

Primers were designed to amplify each of the 30 coding exons of the *ABCA3* gene (see Supplementary Appendix 1, available with the full text of this article at www.nejm.org), and the purified polymerase-chain-reaction products spanning the exons and their respective splice junctions were sequenced on both strands with the use of ABI BigDye Terminator sequencing reagents (Applied Biosystems) and an ABI 3730 sequencer (Applied Biosystems). The results were analyzed with the use of

both SeqMan software (DNASTar) and Mutation Explorer software (SoftGenetics). Variants were identified by comparing each sequence with the reference *ABCA3* sequence.¹⁸ Parental DNA was sequenced when samples were available, and nonsynonymous mutations were analyzed in at least 100 racially or ethnically matched subjects (200 chromosomes). None of the nonsynonymous mutations were found in either the public data base of single-nucleotide polymorphisms (<http://www.ncbi.nlm.nih.gov/SNP/>) or the Celera data base, which is made up of sequences from two European Americans, one African American, one Mexican American, and one Chinese subject.

PHYLOGENETIC ANALYSIS

To analyze the evolutionary (phylogenetic) relation between *ABCA3* and related proteins from differ-

ent organisms, we used the deduced amino acid sequence of ABCA3 (GenBank accession number NP_001080) to search the sequence data base using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>). Similar proteins were also identified in other vertebrate as well as invertebrate species. The amino acid sequences were aligned with the use of the Clustal X program.¹⁹ The alignment was used for phylogenetic analyses involving the Mega2 program (<http://www.megasoftware.net/>).²⁰ This method generates a dendrogram (or tree) indicating the extent to which the sequences are evolutionarily related. To test the reliability of the tree, a bootstrap test with 1000 replications was implemented in the Mega2 program. In this test, the same number of amino acids as in the original data set are randomly sampled from the set of sequences and analyzed. The percentage of the times each branch of the tree has the same topology as the original set of sequences is reported. A bootstrap value of 95 percent or higher provides strong supporting evidence of an evolutionary relation between the particular branch of the tree and the original set of sequences.

ULTRASTRUCTURAL ANALYSIS

Tissue for electron microscopy was fixed in modified Karnovsky's fixative (2 percent paraformaldehyde plus 2 percent glutaraldehyde in 0.1 M sodium cacodylate buffer), post-fixed with 1 percent osmium tetroxide, stained en bloc with cold 4 percent uranyl acetate to preserve the lamellar-body phospholipids, and embedded in EMBed 812 (Electron Microscopy Services) as described previously.²¹ Plastic sections that were 1 μm thick were stained with 1 percent toluidine blue (in 1 percent sodium borate in water) and assessed with the use of a wide-field microscope (Nikon FXA-Microphot). Plastic sections that were 0.1 μm thick were cut from the same blocks as the semithin sections, stained with uranyl acetate and lead citrate, and photographed with a transmission electron microscope (Jeol 1230, Jeol).

RESULTS

DNA SEQUENCING OF ABCA3

To test the hypothesis that the ABCA3 gene is mutated in some infants with surfactant deficiency, we sequenced each of the coding exons of the gene and the flanking splice sites in samples from 21 infants. Polymorphisms identified in this study were first

used to assess the concordance of the six pairs of siblings for ABCA3 haplotypes. One pair of siblings could be excluded from the analysis of recessive mutations in ABCA3, since the siblings were discordant for ABCA3 haplotypes (Fig. 1). The remaining five pairs of siblings were concordant for ABCA3 haplotypes, and the affected infants from consanguineous families were all homozygous for ABCA3 haplotypes.

Mutations were identified in the ABCA3 gene in 16 of the 21 infants (76 percent) (Fig. 2 and Table 2). These included homozygous nonsense mutations in codons 106 and 1142, a homozygous frameshift mutation, and heterozygous insertion mutations and splice-site mutations. Seven missense mutations were identified in conserved amino acids (Fig. 2), including homozygous substitutions of proline for leucine in codons 101 and 1553 (L101P and L1553P, respectively) and heterozygous substitutions of aspartic acid for asparagine at position 568 (N568D), proline for leucine at position 982 (L982P), serine for glycine at position 1221 (G1221S), proline for leucine at position 1580 (L1580P), and proline for glutamine at position 1591 (Q1591P). These missense alleles were not found in control subjects. Several polymorphisms in the introns and exons were also identified (Table 2). The N568 residue is within the N-terminal ATP-binding domain and is conserved in the mammali-

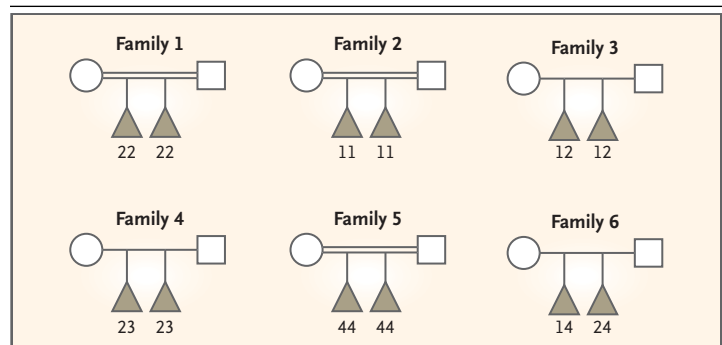


Figure 1. Pedigrees of Patients with Surfactant Deficiency.

Solid symbols indicate patients. The haplotype of ABCA3 polymorphisms is shown below each child (triangular symbols) in the pedigree. In Family 6, the two siblings are discordant for ABCA3 haplotypes, ruling out this gene as the cause of the disorder. All other families have at least one mutation identified in the gene. The haplotype is composed of the following polymorphisms: exon 10–20C/T, F353F(1058C/T), exon 14+33G/A and P585P(1755C/G). Haplotype 1 is C-C-A-C for these polymorphisms in the order given. Haplotype 2 is C-C-G-C, haplotype 3 is T-T-G-G, and haplotype 4 is C-C-G-G. Double lines indicate consanguinity. Circles denote female family members, and squares male family members.

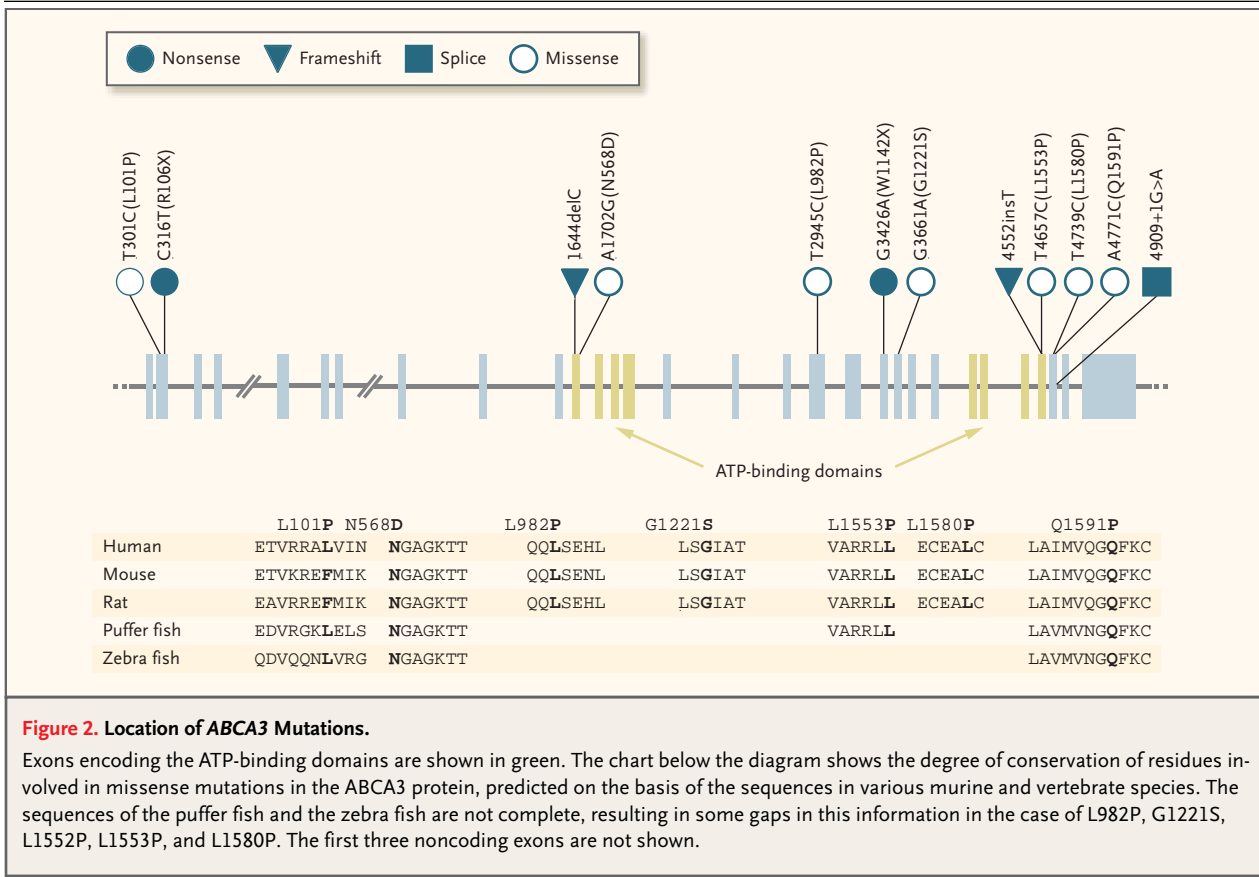


Figure 2. Location of ABCA3 Mutations.

Exons encoding the ATP-binding domains are shown in green. The chart below the diagram shows the degree of conservation of residues involved in missense mutations in the ABCA3 protein, predicted on the basis of the sequences in various murine and vertebrate species. The sequences of the puffer fish and the zebra fish are not complete, resulting in some gaps in this information in the case of L982P, G1221S, L1552P, L1553P, and L1580P. The first three noncoding exons are not shown.

an and fish *ABCA3* genes as well as almost all other members of the ABC type A subfamily (Fig. 2). The corresponding residue is mutated in *ABCA1* in patients with Tangier disease and in *ABCA4* in patients with Stargardt's disease.

ULTRASTRUCTURAL ANALYSIS

Histologic findings in the nine patients with *ABCA3* mutations from whom lung tissue was obtained (Table 1) included hyperplasia of alveolar type II cells, accumulations of alveolar macrophages in distal air spaces with various amounts of proteinaceous material and interstitial thickening, findings consistent with the presence of infantile desquamative interstitial pneumonitis, and neonatal alveolar proteinosis. Plastic sections that were 1 μm thick and stained with toluidine blue were obtained from four patients (Patients 1, 8, 9, and 21), and light-microscopical examination demonstrated alveolar type II cells with homogeneous cytoplasm, without the typical inclusions of lamellar bodies. Electron micrographs of lung tissue from Patient 21 (who was ho-

mozygous for the 4909+1G>A mutation) revealed lamellar bodies (Fig. 3) that were smaller than those from control lung tissue, with more densely packed membranes and eccentrically placed, dense inclusion bodies, similar to those previously described in Patients 1 and 2, who were homozygous for the W1142X mutation.¹⁷ Similarly abnormal lamellar bodies were observed in lung tissue from Patient 8 (who was heterozygous for the G1221S and L982P mutations) and Patient 9 (who was homozygous for the L1553P mutation).

EVOLUTIONARY ANALYSIS OF ABCA3

A missense variant may be a benign polymorphism instead of a deleterious mutation. The variant is more likely to be a deleterious mutation if it is absent in controls and if it affects an amino acid residue that is conserved across species. We therefore aligned the human, mouse, and rat amino acid sequences of *ABCA3* with partial *abca3* sequences of the puffer fish (*Takifugu rubripes*) and zebra fish (*Danio rerio*). Nearly all the missense mutations we iden-

tified occur in residues that are highly conserved (Fig. 2). The amino acid alignment was used to produce a phylogenetic tree of the ABCA3-related proteins showing the relation of the proteins from different organisms (see Supplementary Appendix 2, available with the full text of this article at www.nejm.org). The fish ABCA3 proteins cluster with the mammalian ABCA3 proteins and are distinct from other, more distant ABCA-family proteins, such as the mouse Abca14, Abca15, and Abca16 proteins and the sea-urchin ABCA proteins (see Supplementary Appendix 2. These latter genes are all expressed exclusively in testes and are therefore distinct from ABCA3 genes in both structure and expression.

DISCUSSION

We have demonstrated that the ABCA3 gene is frequently mutated in patients with severe neonatal lung disease and symptoms of surfactant deficiency. Our patients were from several major racial or ethnic groups and our findings therefore indicate that such mutations are not confined to a single group. Most of our patients had mutations predicted to inactivate the gene or protein and died shortly after birth. Electron micrographs of patients' lung tissue demonstrated abnormal lamellar bodies, a finding that is consistent with a role of ABCA3 in the formation of lamellar bodies. All the infants presented with clinical and radiographic findings of surfactant deficiency. Since ABCA3 is related to other transporters of phospholipids and cholesterol, our findings suggest that ABCA3 transports phospholipids that are critical for surfactant function into lamellar bodies. Defective transport of one or more components would be expected to lead to ineffective assembly of the structure and abnormal surfactant. Alternatively, ABCA3 could transport lipids that are deleterious to the function of surfactant out of lamellar bodies.

Missense variants in conserved amino acids were identified in some patients. We were unable to find these same variants in the public polymorphism data bases or in 100 racially or ethnically matched controls, but in the absence of a functional test, we cannot rule out the possibility that these are neutral variants. In the case of the L101P and L1553P mutations, each of which affected one pair of siblings from two different families, the two pairs of siblings were both homozygous for the variant and homozygous for all other polymorphisms that we found in the gene — findings that are consistent

Table 2. Mutations and Polymorphisms Identified in the ABCA3 Gene.

Variant	Patient No.	Nucleotide Affected*	Site Affected or Outcome	SNP No.†
Mutation				
Exon 5	3, 4	T301C	L101P‡	
Exon 5	14	C316T	R106X‡	
Exon 14	20	A1702G	N568D	
Exon 14	13	1644delC	Frameshift‡	
Exon 21	7, 8	T2945C	L982P	
Exon 23	1, 2	G3426A	W1142X‡	
Exon 24	7, 8	G3661A	G1221S	
Exon 30	9, 10	T4657C	L1553P	
Exon 30	5, 6	4552insT	Frameshift	
Exon 31	15, 21	4909+1G>A	Splice site‡	
Exon 31	5, 6	T4739C	L1580P	
Exon 31	19	A4772C	Q1591P	
Polymorphism				
Exon 5	Multiple	Exon 5+50A/G	Intron	rs46725
Exon 6	20	393C/T	A131A	
Exon 6	18	Exon 6+119G/A	Intron	rs323059
Exon 7	19	Exon 7-14C/G	Intron	
Exon 8	14	681C/T	A227A	
Exon 10	Multiple	Exon 10-105C/A	Intron	rs323066
Exon 10	Multiple	Exon 10-20C/T	Intron	
Exon 10	Multiple	1058C/T	F353F	
Exon 14	Multiple	Exon 14+33G/A	Intron	rs170447
Exon 15	Multiple	1755C/G	P585P	rs323043
Exon 18	13	Exon 18-17G/A	Intron	
Exon 18	1	2340C/T	H780H	
Exon 21	Multiple	Exon 21-20C/G	Intron	rs313908
Exon 21	Multiple	Exon 21+34C/T	Intron	rs313909
Exon 27	Multiple	4116C/T	S1372S	rs149532
Exon 32	11	4944C/T	V1648V	

* Nucleotides are numbered from the ATG start codon.
 † Polymorphisms with entries in the public data base of single-nucleotide polymorphisms (SNPs) (<http://www.ncbi.nlm.nih.gov/SNP/>) are indicated.
 ‡ This sample was homozygous for the mutation.

with the occurrence of a recessive mutation in these consanguineous families. The N568D mutation is in a highly conserved residue in the ATP-binding domain, and it almost certainly disrupts the function of the protein. The clinical phenotype and histopathological findings in patients with missense mutations were similar to those in patients with apparent loss-of-function mutations, supporting the notion that the mutations were deleterious rather than neutral variants.

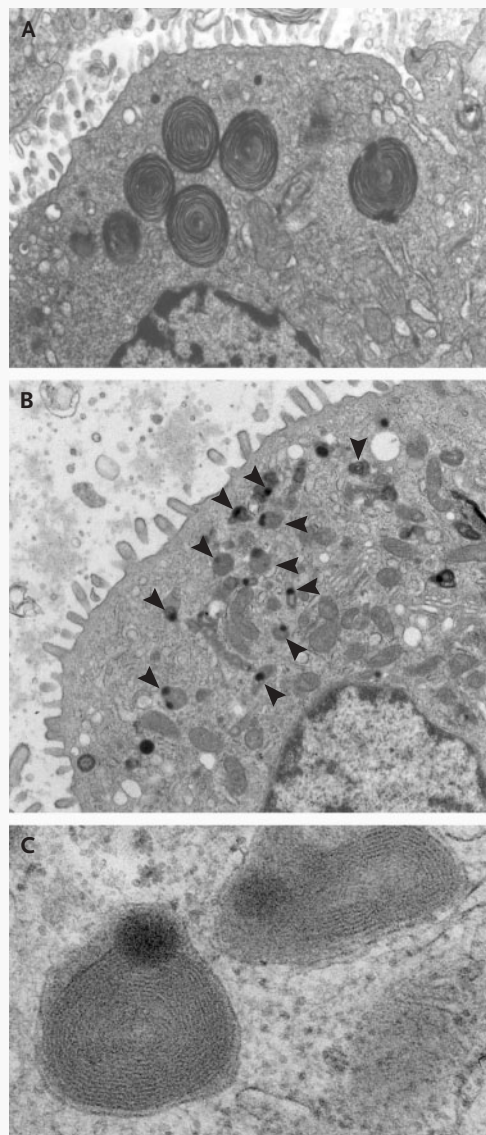


Figure 3. Ultrastructure of Alveolar Type II Cells.

A representative electron micrograph of normal lung tissue shows normal lamellar bodies (Panel A, $\times 15,000$). In lung tissue from Patient 21, who was homozygous for an *ABCA3* splicing mutation (4909+1G>A), cytoplasmic lamellar bodies are smaller and denser (arrowheads in Panel B, $\times 15,000$), and many have dense peripheral inclusions (Panel C, $\times 80,000$).

In two patients, a mutation was identified on only one allele. Although the *ABCA3* sequence variants in these children may have been unrelated to their lung disease, the similarity of their clinical presentation and the severe nature of their lung disease

suggest that these children probably had a second mutation on the other allele, which may have been mutations within introns or regulatory regions or large rearrangements or deletions. These variations would not have been detected by our sequencing strategy. Similar rates of detection of mutations have been reported among patients with mutations in the genes for other ABC transporters such as the cystic fibrosis transmembrane conductance regulator and *ABCA4*.^{22,23}

These findings indicate that families in which mutations are identified may benefit from genetic counseling and prenatal or preimplantation diagnoses. We found different mutations in the different families, suggesting that there are no common alleles that confer this condition. One patient, who had a missense mutation (Q1591P) on one allele and an unknown mutation on the other allele, is still alive at six years of age and has chronic lung disease, suggesting that some *ABCA3* mutations are not fatal. *ABCA3* is thus a candidate gene for other pulmonary disorders involving surfactant dysfunction, including the neonatal respiratory distress syndrome and disorders with a later onset, such as asthma and the acute respiratory distress syndrome. There is considerable heterogeneity in the presentation and severity of the respiratory distress syndrome in premature infants, and different *ABCA3* variants could influence the severity of that condition. We did not find many nonsynonymous variants in *ABCA3* that represent candidate single-nucleotide polymorphisms for the respiratory distress syndrome and other disorders. However, the *ABCA3* gene is hormonally regulated,^{13,14} and genetic variants (in other genes) that indirectly affect its regulation might also be important.

ABCA3 mutations were identified in 16 of our 21 patients (76 percent), and 3 of the 5 patients without *ABCA3* mutations recovered completely from their initial lung disease and thus did not have the identical phenotype. The high percentage of patients with *ABCA3* mutations in the group of 21 infants suggests that *ABCA3* deficiency may account for a substantial number of cases of fatal lung disease among full-term infants for which no specific cause can be identified, but further study is needed to address the relative contributions of mutations in *SFTPB*, *SFTPC*, and *ABCA3* to neonatal lung disease. The absence of mutations in some full-term infants with fatal surfactant deficiency indicates that other, as yet unidentified genes are essential for surfactant production. This possibility is not surpris-

ing, given the complex nature of surfactant and the many different types of proteins in lamellar bodies.

We observed distinct ultrastructural changes in lamellar bodies in association with *ABCA3* mutations, and abnormal lamellar bodies were also seen in association with a deficiency of surfactant protein B.²⁴ These findings illustrate the potential importance of electron microscopy in the examination of lung tissue from infants who are dying from a lung disease of unclear causation.

The *ABCA3* gene is highly conserved in both mammals and fish, suggesting that its role in the production of surfactants predates the development of the lung. This possibility is consistent with morphologic studies showing that the surfactants

in the swim bladders of teleost (bony) fish contain phospholipids and proteins similar to those found in the mammalian lung.²⁵ Surfactants are also found in the airways of reptiles, salamanders, and lungfish. The conservation of the *ABCA3* gene across diverse vertebrate species supports a role of the *ABCA3* protein in surfactant lipid metabolism and cellular homeostasis, although the protein may have other functions as well.

Supported by grants from the National Institutes of Health (HL-54703, to Dr. Noguee, and HL-56387, to Drs. Wert, Noguee, and Whitsett) and from the Eudowood Foundation (to Dr. Noguee).

We are indebted to Bernard Gerrard and Georgianne Cirado for technical assistance, to the families who participated in these studies, to the physicians and nurses who cared for the children, and to Henry Shuman for helpful suggestions.

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