0031-3998/07/6202-0176
PEDIATRIC RESEARCH

Vol. 62, No. 2, 2007
Printed in U.S.A.

Heterozygosity for *ABCA3* Mutations Modifies the Severity of Lung Disease Associated with a Surfactant Protein C Gene (*SFTPC*) Mutation

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ABSTRACT: Heterozygous *SFTPC* mutations have been associated with adult and pediatric interstitial lung disease (pILD). Inheritance is autosomal dominant, but *de novo* mutations may cause sporadic disease. *SFTPC* mutations have been associated with variable onset of symptoms, ranging from early infancy to late adulthood. The underlying mechanisms for this variability are unknown. Recently, mutations in *ABCA3* (encoding member A3 of the adenosine triphosphate–binding cassette family of transporters) were identified as a cause of pILD. To test the hypothesis that *ABCA3* mutations modify the severity of lung disease in individuals with *SFTPC* mutations, we sequenced *ABCA3* from four symptomatic infants with the same *SFTPC* mutation, a substitution of isoleucine by threonine in codon 73 (I73T). Each infant developed respiratory symptoms by 2 mo of age and inherited the mutation from an asymptomatic parent. Three of the four infants were also heterozygous for an *ABCA3* mutation, which was inherited from the parent without *SFTPC* I73T. The finding of heterozygosity for *ABCA3* mutations in severely affected infants with *SFTPC* I73T, and independent inheritance from disease-free parents supports that *ABCA3* acts as a modifier gene for the phenotype associated with an *SFTPC* mutation. (***Pediatr Res* 62: 176–179, 2007**)

Pulmonary surfactant is a mixture of lipids and specific proteins that reduces surface tension at the air-liquid interface and prevents end-expiratory atelectasis. Lack of surfactant is the primary cause of respiratory distress syndrome (RDS) in infants born prematurely. Although surfactant phospholipids are primarily responsible for lowering surface tension, two small hydrophobic proteins, surfactant proteins B and C (SP-B and SP-C), have key roles in regulating surfactant function and metabolism (1). SP-C deficiency and mutations in the SP-C gene (*SFTPC*) have been associated with both sporadic interstitial lung disease (ILD) due to *de novo* mutations and familial ILD inherited as autosomal dominant (2–10). One particular *SFTPC* mutation, a substitution of threonine (T) for isoleucine (I) in codon 73 (I73T) has been identified in multiple unrelated families (9–11). The age at onset and severity of lung disease due to *SFTPC* I73T as well as other *SFTPC* mutations is highly variable, ranging from death in early infancy to development of pulmonary fibrosis in the fifth or sixth decade (7, 9–12). SP-C deficiency in knockout mouse models results in lung disease depending on genetic background (13,14). The variability in mouse and human lung disease suggests that *SFTPC*-associated lung disease may be influenced by environmental factors and/or modifier genes.

A candidate gene that may modify *SFTPC*-related lung disease is the ATP-binding cassette A3 (*ABCA3*) gene. *ABCA3* encodes a 1704-amino acid protein that is highly expressed in the lung and has been localized to the limiting membrane of lamellar bodies in alveolar type II cells (15–18). Recent studies support that ABCA3 transports lipids into lamellar bodies, the storage organelle for surfactant within alveolar type II cells and is required for the formation of normal lamellar bodies (19–21). Autosomal recessive ABCA3 deficiency results in fatal neonatal RDS (22), whereas some *ABCA3* mutations, also inherited in autosomal recessive fashion, are associated with ILD and prolonged survival (23). The lung histopathology in children with the milder phenotype is similar to that observed in children with *SFTPC* mutations. We hypothesized that *ABCA3* genetic variants modify the pulmonary phenotype of some *SFTPC* mutations.

METHODS

Patients. From June 1993 through December 2005, DNA samples were obtained from 325 children with chronic lung disease of unknown etiology as part of a study to identify genetic defects in surfactant metabolism. The entry criteria included the following: (1) gestational age of 36 wk or older and age 30 d or older or discharged from the neonatal intensive care unit or gestational age younger than 36 wk and age older than 3 mo or discharge from the neonatal intensive care unit and (2) indications of parenchymal lung disease as manifested by two of three of the following factors: need for supplemental oxygen; clinical signs of lung disease, including cough, tachypnea, retractions, or rales; and abnormal chest radiograph. Infants younger than 30 d were eligible for enrollment if there was a family history of lung disease. A history of neonatal lung disease was not a requirement for inclusion. The children were referred by their primary providers. The review boards of the participating institutions approved the protocols for these evaluations, and written consent for genetic testing was obtained from the families. Patients were not excluded based on sex or race/ethnicity. Fifty-six percent was male, 43% female, and 1% unknown. The distribution of samples by racial/ethnic background was 218 (67%) white (non-Hispanic), 23 (7%) African American, 41 (12.5%) Hispanic, 15 (4.5%) Asian, 6 (1.8%) other, and 17 (5.2%) unknown.

DNA preparation and mutational analysis. Genomic DNA was prepared from blood leukocytes with use of a commercially available kit (Gentra Systems, Minneapolis, MN). Polymerase chain reaction (PCR) products spanning exons 1 and 2 (genomic positions, –143 to 996) and exons 3 to 6

Received January 2, 2007; accepted March 23, 2007.
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Supported by the National Institutes of Health (HL-54703 to L.M.N.) and the Eudowood Foundation.

Abbreviations: ***ABCA3*,** adenosine triphosphate–binding cassette protein group A3 gene; **pILD,** pediatric interstitial lung disease; ***SFTPC,*** surfactant protein C gene

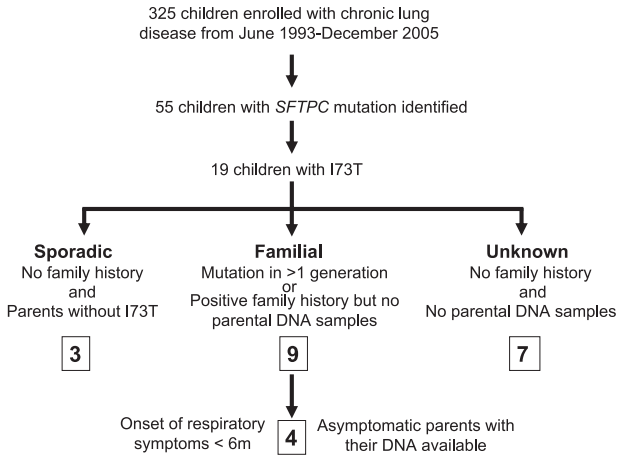

Figure 1. Flow table for selection of children with chronic lung disease and *SFTPC* I73T for *ABCA3* gene analysis. Seven of these patients were the subject of earlier reports.

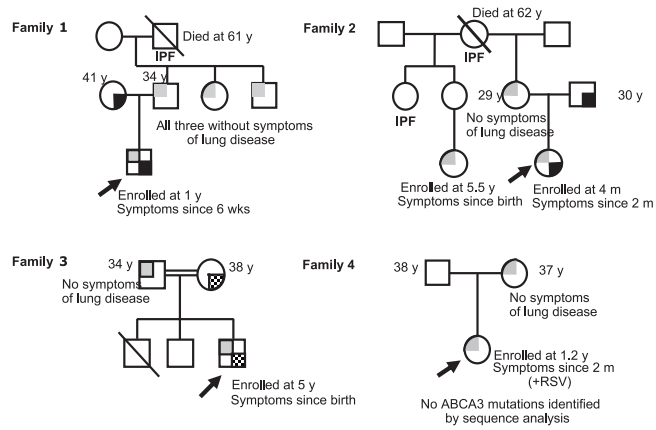

Figure 2. Pedigrees of patients with *SFTPC* I73T evaluated for *ABCA3* mutations. Index cases identified by *arrows* with age at enrollment and onset of symptoms noted below symbol. Age of parents and symptomatic family members are noted below symbol. *Circle*, female; *square*, male; *shaded quarter symbol*, *SFTPC* I73T; *solid quarter symbol*, *ABCA3* E292V; *checkered quarter symbol*, *ABCA3* L212M; *slash through symbol*, deceased; IPF, idiopathic pulmonary fibrosis; RSV, respiratory syncytial virus.

(genomic positions, 1212 to 2522) of the *SFTPC* gene were generated from genomic DNA by PCR and analyzed by direct sequencing of the PCR products with the use of previously described conditions (24). The 30 coding exons of the *ABCA3* gene and their respective splice junction sites were amplified by previously published conditions and primer sets (23). Automated DNA sequencing was performed through the Johns Hopkins School of Medicine's genetic resources core DNA sequencing facility using Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). DNA sequencing chromatograms were analyzed with the aid of Sequencher 4.2 software (Gene Codes Corporation, Ann Arbor, MI). The resulting *ABCA3* and *SFTPC* sequences were compared with reference sequences (15,25).

Restriction digest analysis. The restriction endonuclease *BsrGI* was purchased from New England Biolabs (Beverly, MA) and used according to manufacturer's specifications for analysis of *ABCA3* E292V, as reported previously (23).

RESULTS

Of the 325 children enrolled in the study, 55 (17%) were found to have an *SFTPC* mutation identified as the probable cause of their lung disease. *SFTPC* I73T was the most commonly identified mutation, found in 19 children, which comprised 35% of the mutant *SFTPC* alleles. Seven of the 19 children were the subject of an earlier report, including one who was a compound heterozygote for *SFTPC* I73T and *SFTPC* L110R (10). *ABCA3* E292V was assayed for in the 19 children because of its association with multiple unrelated children and lung disease (23). Two infants were identified with *ABCA3* E292V and had the onset of their symptoms at younger than 2 mo of age. Each parent did not have any respiratory symptoms, and DNA samples were available from both parents for analysis (Fig. 1). Two additional infants were discordant in their respiratory symptoms from their parents and also had available DNA from the both of their parents (Table 1). The 30 coding exons of *ABCA3* were sequenced from these four children. Sequencing confirmed that patients 1 and 2 had the *ABCA3* E292V mutation and no other mutation. Patient 3 was heterozygous for a missense mutation in exon 8, a substitution of methionine for leucine at codon 212, L212M. Patient 4 did not have any mutations identified. Analysis of parental DNA of three children who were heterozygous for both *SFTPC* I73T, and an *ABCA3* mutation demonstrated that the *ABCA3* mutation was inherited from the opposite parent who had *SFTPC* I73T (Fig. 2).

DISCUSSION

There are many examples of monogenic disorders whose phenotype is influenced by modifier genes, such as polymorphisms that affect the expression of fetal Hb in sickle cell disease (26). We chose *ABCA3* as a candidate modifier gene for the lung disease due to *SFTPC* mutations because of its involvement in surfactant metabolism and the similarities in the lung pathology findings in children with *ABCA3* mutations to those with *SFTPC*-related lung disease. To test this hypothesis, we selected families with the most severe pulmonary phenotypic discordance, each with an affected infant whose respiratory symptoms began at younger than 2 mo of age and an asymptomatic parent with *SFTPC* I73T. We focused on families with *SFTPC* I73T, as it is the most frequently re-

Table 1. *Characteristics of infants with SFTPC I73T and asymptomatic parents*

	Patient 1	Patient 2	Patient 3	Patient 4
Race	White	White	White, Middle Eastern descent	White
Age at symptoms	6 wk	Birth (TTN)	Birth	Birth (TTN)
Sex	Male	Female	Male	Female
Age at enrollment	13 mo	4 mo	5 yo	14 mo
Lung biopsy	Not done	Not done	Cholesterol pneumonitis with mild fibrosis	Not done
Current age	30 mo	22 mo	7 y	25 mo
Family history	Yes	Yes	Yes	None

TTN, transient tachypnea of newborns.

ported *SFTPC* mutation associated with lung disease, (9–12) and to minimize effects on disease severity due to different *SFTPC* mutations. Three of four infants were also heterozygous for an *ABCA3* mutation, supporting a role for *ABCA3* in modifying lung disease due to *SFTPC* mutations.

Two of the four infants had *ABCA3* E292V, a mutation that has been associated with pILD (23). The population frequency of *ABCA3* E292V is low, approximately one in 275 individuals (personal communication, T. Garmany and A. Hamvas). Thus, the finding of *ABCA3* E292V in two of the 19 patients with *SFTPC* I73T seems unlikely to have occurred by chance. The third infant had a different missense mutation, *ABCA3* L212M, which results in a conservative neutral substitution. It is possible that L212M represents a rare but benign polymorphism. However, two siblings with significant respiratory symptoms and who had lung histopathology findings consistent with surfactant dysfunction were compound heterozygotes for a previously identified *ABCA3* nonsense mutation (R106X) and L212M, suggesting that L212M is a disease-causing mutation (L.M. Nogee, unpublished data). No *ABCA3* mutation was identified in the fourth infant. Other genetic modifiers or other critical environmental exposures may have contributed to the severity of lung disease, in particular a respiratory syncytial virus infection at 2 mo of age. A history of viral infections was noted in symptomatic young infants in a large kindred with familial ILD due to *SFTPC* L188Q (7). In addition, RSV infection of cells in culture that expressed a low level of a *SFTPC* mutation identified in patients with ILD that skips exon 4 (*SFTPC* Δexon4) resulted in cell death, indicating a possible interaction of low levels of expression of abnormal SP-C with viral infections (27).

The pathophysiology of *SFTPC*-related lung disease is complex, and the mechanisms whereby *ABCA3* mutations modify the lung disease due to *SFTPC* mutations are unknown. *SFTPC* mutations may cause SP-C deficiency by a dominant negative mechanism. Mature SP-C is derived from a larger proprotein (proSP-C) that self-associates in the secretory pathway (28). ProSP-C containing mutations may be targeted for degradation (29). Thus, abnormal SP-C may also cause wild-type SP-C to be degraded, leading to SP-C deficiency. Decreased or absent SP-C has been observed in some patients with *SFTPC* mutations (3,24,30). As ABCA3 deficiency alters surfactant homeostasis, including abnormal processing and routing of SP-C (31) and hindering lamellar body biogenesis (19), decreased functional ABCA3 activity in patients with *SFTPC* mutations may result in more severe symptoms of surfactant deficiency. We did not perform expression studies to directly assess the effects of the *ABCA3* mutations observed in these patients.

Lung disease due to *SFTPC* mutations may result from a toxic gain-of-function mechanism of abnormal proSP-C. Increased proSP-C expression may overwhelm the protein degradation pathway leading to abnormal accumulations of proSP-C containing mutations. Perinuclear accumulations of mutated proSP-C have been observed *in vitro* (32,33) and in explanted lung tissue from a patient with an *SFTPC* mutation (6). These accumulations of abnormal protein may result in the unfolded protein response, cytotoxicity, and eventual apoptotic death (5,34). Expression of *SFTPC* Δexon4 in transgenic mice disrupted lung development that correlated with the degree of transgene expression, further supporting the potential toxicity of abnormal proSP-C (29). Altered surfactant homeostasis with abnormal processing and routing of SP-C due to decreased ABCA3 function (31) may thus exacerbate the accumulation of inappropriately processed SP-C, leading to more cell injury and death. Both loss-of-function and toxic gain-of-function mechanisms may be involved in the development of *SFTPC*-related lung disease at different points in time.

The relative contribution of *ABCA3* to *SFTPC*-related lung disease is unknown. Our study was not population based with enrolled infants identified by their primary physicians based on the unusual nature of their lung disease, and we selected those infants for whom we had parental DNA and whom knew had variable severity of lung disease. There is thus a significant ascertainment bias that limits interpretation of the frequency of *ABCA3* as a gene that modifies some *SFTPC* mutations.

In summary, the finding that three infants with severe lung disease and *SFTPC* I73T were also heterozygous for *ABCA3* mutations, in contrast to their asymptomatic parents from whom they inherited the *SFTPC* mutation, supports the hypothesis that *ABCA3* mutations modify the severity of lung disease associated with *SFTPC* mutations. Studies of the effects of *SFTPC* mutations in combination with *ABCA3* mutations both *in vitro* and ultimately in animal models will be needed to confirm this hypothesis. We speculate that *ABCA3* genetic variants may also contribute to the development and progression of other common diseases in the general population, such as asthma or bronchopulmonary dysplasia.

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