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H. 知的財産権の出願・登録状況

5. 特許取得

なし

6. 実用新案登録

なし

7. その他

なし

III. 研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

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Molecular and cellular characteristics of *ABCA3* mutations associated with diffuse parenchymal lung diseases in children

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***ABCA3* (ATP-binding cassette subfamily A, member 3) is expressed in the lamellar bodies of alveolar type II cells and is crucial to pulmonary surfactant storage and homeostasis. *ABCA3* gene mutations have been associated with neonatal respiratory distress (NRD) and pediatric interstitial lung disease (ILD). The objective of this study was to look for *ABCA3* gene mutations in patients with severe NRD and/or ILD. The 30 *ABCA3* coding exons were screened in 47 patients with severe NRD and/or ILD. *ABCA3* mutations were identified in 10 out of 47 patients, including 2 homozygous, 5 compound heterozygous and 3 heterozygous patients. SP-B and SP-C expression patterns varied across patients. Among patients with *ABCA3* mutations, five died shortly after birth and five developed ILD (including one without NRD). Functional studies of p.D253H and p.T1173R mutations revealed that p.D253H and p.T1173R induced abnormal lamellar bodies. Additionally, p.T1173R increased IL-8 secretion *in vitro*. In conclusion, we identified new *ABCA3* mutations in patients with life-threatening NRD and/or ILD. Two mutations associated with ILD acted via different pathophysiological mechanisms despite similar clinical phenotypes.**

INTRODUCTION

Pulmonary surfactant, a complex mixture of lipids and specific proteins located at the air–liquid interface, lowers alveolar surface tension, thereby preventing alveolar collapse at the

end of expiration. It is synthesized by alveolar type-II cells, stored in lamellar bodies and secreted by exocytosis. Phospholipids make up ~90% of pulmonary surfactant.

Recent studies indicate a role for several genes in diffuse lung diseases (1–3). Genes implicated to date include the

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Table 1. Genetic analysis results in the 10 children harboring homozygous and compound heterozygous (shaded) or heterozygous *ABCA3* mutations

Patient	NRD	Clinical outcome	<i>ABCA3</i> mutation cDNA level	Protein level	<i>ABCA3</i> SNPs dbSNPs rs# cluster id	<i>ABCA3</i> variants Missense variants in conserved amino acid
1	Yes	ILD	c.[3518C>G] + [3518C>G]	p.[T1173R] + [T1173R]	rs149532, rs13332514	
2	Yes	ILD	c.[757G>C] + [757G>C]	p.[D253H] + [D253H]		
3	Yes	Death	c.[1385T>G] + [2890G>A]	p.[L462R] + [G964S]	rs149532	
4	Yes	Death	c.[4747C>T] + c.[384delC]	p.[R1583W] + p.[S128Rfs]	rs149532	c.[450G>A] (het)
5	No	Death	c.[629G>T] + [3079G>C]	p.[G210V] + [A1027P]	rs149532	
6	Yes	ILD	c.[622C>T] + [4561C>T]	p.[R208W] + [R1521W]	rs149532, rs323043	
7	Yes	Death	c.[604G>C] + [907C>G]	p.[G202R] + [L303V]	rs149532, rs323043 (het), rs13332514	
8	Yes	Death	c.[2888A>G] + [?]	p.[Y963C] + [?]	rs149532 (het), rs323043 (het)	
9	Yes	ILD	c.[2125C>T] + [?]	p.[R709W] + [?]	rs149532	
10	Yes	ILD	c.[2614A>G] + [?]	p.[S872G] + [?]	rs149532 (het), rs323043, rs13332514 (het)	

het, heterozygous; NRD, neonatal respiratory distress; ILD, interstitial lung disease.

surfactant protein (SP)-B and SP-C genes (*SFTPB*, MIM 178640; and *SFTPC*, MIM 178620) and the ATP-binding cassette subfamily A member 3 gene (*ABCA3*, MIM 601615). SP-B deficiency has long been known to cause lethal neonatal respiratory distress (NRD) (4). More recently, *SFTPC* mutations were reported in newborns and infants with severe alveolar-interstitial syndrome (3,5). *ABCA3* is a 1704-amino acid protein expressed selectively—but not specifically—in the lung, where it is found in the limiting membrane of lamellar bodies (1,6,7). *ABCA3* is encoded by an 80 kb gene mapped to 16p13.3 in humans and is thought to regulate lipid transport and organization during lamellar body formation (8,9).

ABCA3 gene mutations are transmitted by autosomal recessive inheritance. As with SP-B deficiency, *ABCA3* deficiency should be suspected in full-term infants with severe NRD refractory to maximal conventional treatment (10,11). In addition, *ABCA3* gene mutations have been found in children and young adults with interstitial lung disease (ILD) (1,3,12). For instance, the heterozygous c.875A>T (p.Glu292Val, p.E292V) *ABCA3* mutation was identified in several older children and young adults with desquamative interstitial pneumonitis (1). The large size and marked allelic heterogeneity of the *ABCA3* gene create challenges in mutation identification.

The objectives of this study were to identify and characterize *ABCA3* variations in a large population of pediatric patients with NRD and/or ILD. We identified new *ABCA3* gene mutations and found that these mutations were not associated with a specific expression profile of SP-B and SP-C in bronchoalveolar lavage fluid (BALF). Functional analysis of two mutations associated with ILD showed different pathophysiological mechanisms, despite the similar clinical phenotype.

RESULTS

Study patients

Of the 47 children enrolled in the study (Supplementary Material, Supporting Information 1), 23 (49%) were male and 24 (51%) female. The patients were from Europe ($n = 27$), North Africa ($n = 12$), Reunion Island ($n = 6$), West Africa ($n = 1$) and Haiti ($n = 1$). Among them, 6 (13%) were born

prematurely (<36 weeks) and 31 (66%) had NRD. ILD developed in 31 (66%) patients, and 21 (36%) patients had both NRD and ILD. Nine (19%) patients died of respiratory failure.

Genetic analysis

Of the 47 patients, 10 had *ABCA3* mutations. We identified 15 mutations, including 13 that had not been described previously. The two mutations p.G210V and p.R208W have been already identified (13,14). There were 14 missense mutations and 1 heterozygous nonsense mutation (p.Ser128ArgfsX23, designated hereafter as p.S128Rfs) (Table 1).

Analysis of genomic DNA from the parents and kindred showed that the compound heterozygous p.R1583W/p.S128Rfs (Fig. 1A) and p.R208W/p.R1521W (Fig. 1B) mutations were inherited, as well as the homozygous mutations p.T1173R (Fig. 1C) and p.D253H (Fig. 1D). For the other mutations, genomic DNA samples from family members were not available.

None of these newly identified *ABCA3* mutations has been previously described as polymorphisms (<http://ncbi.nih.gov/> SNP). In addition, none of the new variants was detected in the 46 alleles from our 23 controls. Alignment of the human and other mammalian amino acid sequences (by Multiple Sequence Comparison using Log-Expectation, MUSCLE analysis) indicated that almost all the *ABCA3* mutations occurred in highly conserved residues (not illustrated). They were located across the protein in the extracellular domains (ECD1 and ECD2), as well as in internal domains (NBD1 and NBD2) (Fig. 2). Finally, complete *ABCA3* sequencing disclosed previously described single-nucleotide polymorphisms (SNPs) (Table 1), as well as a missense variant affecting a conserved amino acid in the patient harboring the c.[4747C>T]+[384delC] mutation.

In the 37 patients without *ABCA3* mutations, four SNPs were identified in the coding region of *ABCA3*. These SNPs were in exons 7, 9, 14 and 26, respectively, and did not induce amino acid variations. A missense variant in the conserved amino acid c.1059C>T was identified in nine children. We found these variants neither in the public polymorphism database nor in our controls.

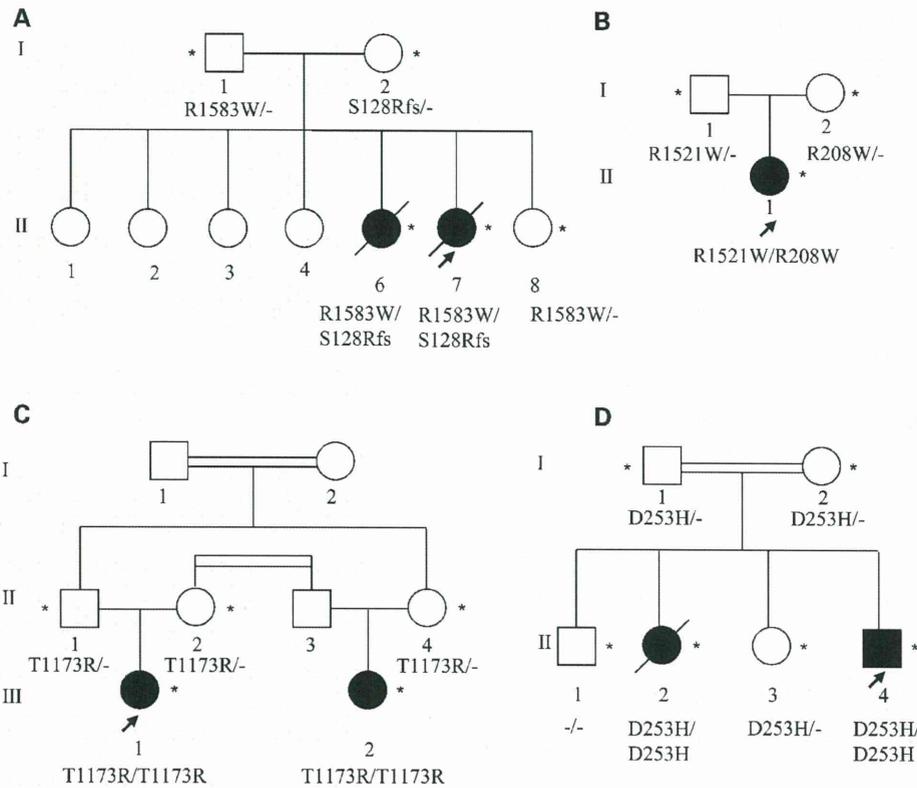


Figure 1. Pedigree of the families with the *ABCA3* mutations p.R1583W/p.S128Rfs (A), p.R1521W/R208W (B), p.T1173R/p.T1173R (C) and p.D253H/p.T1173R (D). Asterisks indicate family members with *ABCA3* mutation analysis, and arrows indicate index patients.

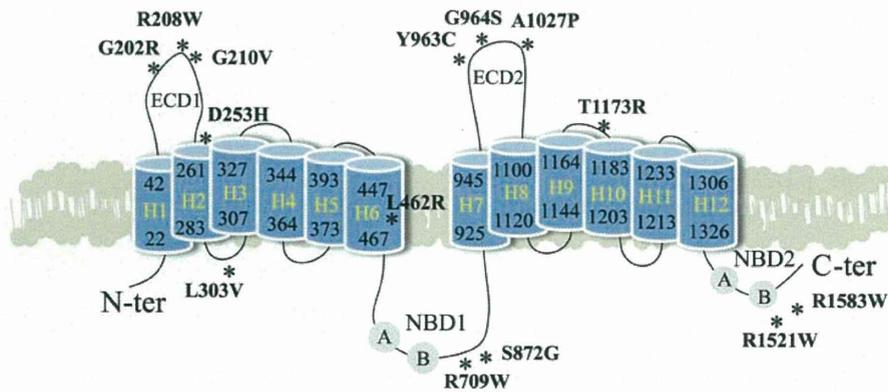


Figure 2. Schematic representation of the *ABCA3* protein [N-terminal (N-ter) to C-terminal (C-ter) domains] and the location of the novel mutations (indicated by an asterisk). The 12 putative transmembrane helix (H1–H12) domains, two extracellular domains (ECD1 and ECD2) and two nucleotide-binding domains (NBD) (including the conserved motifs Walker A and B) are represented.

Characteristics of patients with *ABCA3* mutations

The characteristics of the 10 children with *ABCA3* mutations are reported in Table 2. Among them, nine (90%) had NRD, five progressed to ILD and five died of respiratory failure (all during the first year of life).

Three children harboring homozygous and compound heterozygous mutations who developed ILD (patients 1, 2 and 6) were treated with methylprednisolone pulse for, respectively, 14

months (patient 1), 6 years (patient 2) and 11 years (patient 6, who is still on methylprednisolone pulse). They also received azithromycin for, respectively, 2 years (patient 1), 12 years (patient 2) and 2 years (patient 6), patients 2 and 6 still being treated. Two patients (patients 2 and 6) required prolonged oxygen supplementation for, respectively, 10 years (patient 2) and 11 years (patient 6, who is still on oxygen).

Two children harboring heterozygous mutations developed ILD (patients 10 and 9). The first child (patient 10) was

Table 2. Clinical characteristics of the 10 patients with *ABCA3* mutations

	<i>ABCA3</i> -mutated patients (n = 10)
Sex: male/female, n (%)	4 (40)/6 (60)
Median age at onset in months (range)	0 (0–6)
Neonatal respiratory distress, n (%)	9 (90)
Hypoxemia, n (%)	10 (100)
Physical findings, n (%)	
Tachypnea	10 (100)
Retractions	10 (100)
HRCT findings, n (%)	
Ground-glass opacities	8 (100)
Lung cysts	3 (38)
Interlobular septal thickening	2 (25)
Consolidation	4 (50)
Lung biopsy, n (%)	
Type-II cell hyperplasia	6 (100)
Septal thickening	6 (100)
Mild fibrosis	5 (83)
Intra-alveolar macrophages	6 (100)
Outcome n (%)	
ILD/death	5 (50)/5 (50)

ILD, interstitial lung disease; n corresponds to the number of available patients' clinical data.

treated by monthly methylprednisolone pulse for 3 years and required oxygen supplementation for 8 months. The second one (patient 9) did not receive any treatment during the study period.

All 10 patients had clinical symptoms of respiratory failure. High-resolution computed tomography (HRCT) scans were available for eight patients and predominantly showed ground-glass opacities (Fig. 3A and Supplementary Material, Supporting Information 2). Lung biopsy was performed in six patients, all of whom had alveolar septal thickening, a few interstitial inflammatory cells (polymorphonuclear neutrophils and lymphocytes), uniform prominent type-II cell hyperplasia and accumulation of intra-alveolar macrophages (Fig. 3B). Electron microscopy was performed on a lung biopsy from the patient harboring the p.D253H mutation and showed abnormal lamellar bodies with dense inclusions (Fig. 3C).

BALF analysis

Western blot analysis of surfactant proteins (Fig. 4) was performed in seven patients, who had the following *ABCA3* mutations: p.D253H (patient 2), p.T1173R (patient 1), p.L462R/p.G964S (patient 3), p.G202R/p.L303V (patient 7), p.Y963C (patient 8), p.R1583W/p.S128Rfs (patient 4) and p.S872G (patient 10), respectively. SP-C (Fig. 4A) and SP-B (Fig. 4B) were detected at a size of 3.7 and 8 kDa in variable amounts, the smallest amount being found in the patient with the p.G202R/p.L303V *ABCA3* mutation (patient 7) in whom SP-C and SP-B are only faintly visible. This patient was the only one who cannot be weaned off mechanical ventilation before he died of respiratory failure. Interestingly, the two children harboring homozygous mutations (patients 1 and 2) have small but detectable amounts, which were nonetheless compatible with life. In contrast, patient 4, who died within the first month of life, had amount of SP-B and SP-C similar to control.

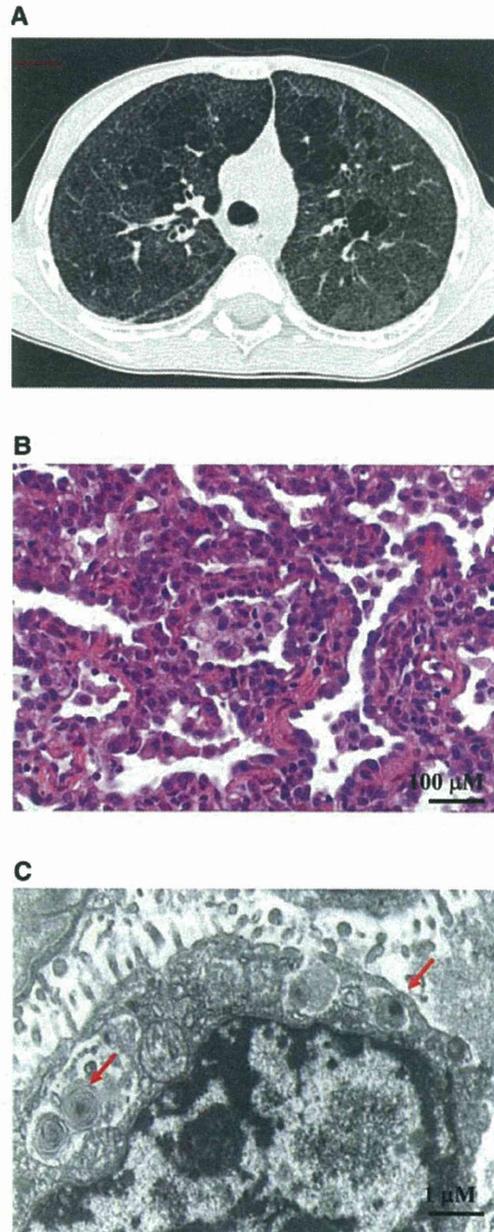


Figure 3. (A) HRCT scan, (B) hematoxylin and eosin (HE) staining of lung tissue and (C) electron microscopy of type-II cells from the patient (patient 2) harboring the *ABCA3* homozygous mutation p.D253H. Red arrows indicate lamellar bodies.

Characterization of *ABCA3* mutations

The two mutations p.T1173R and p.D253H were deliberately chosen for subsequent functional studies because they were homozygous. Also, since past functional studies of *ABCA3* focused mainly on NRD-associated mutations, it was crucial to study the consequences of these two mutations associated with progression towards ILD.

ABCA3 localization and processing. We first investigated the intracellular localization of the mutated *ABCA3* protein.

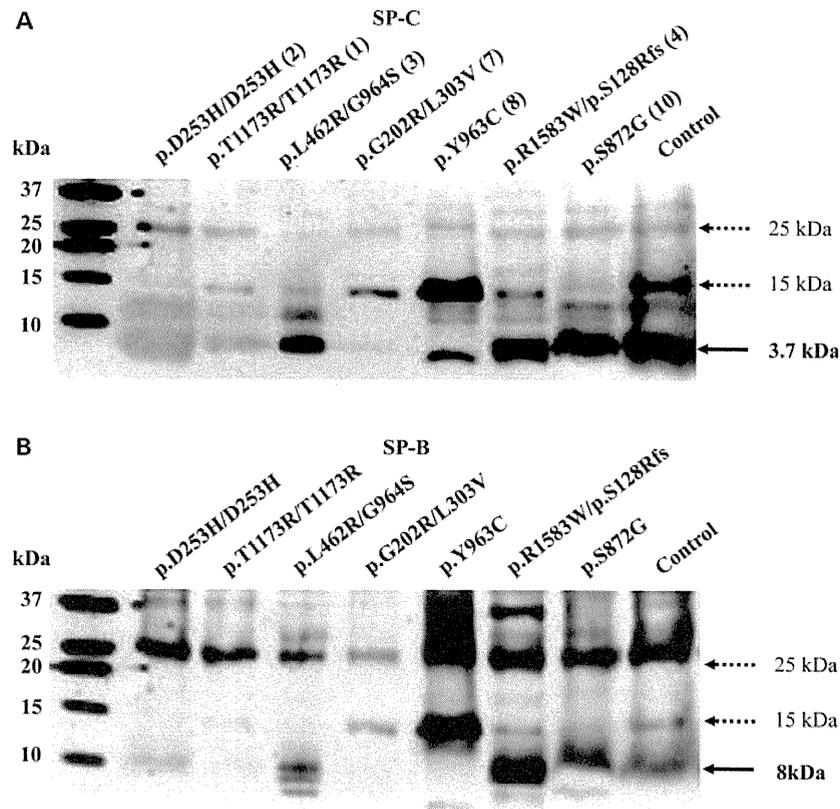


Figure 4. Western blot analysis of surfactant proteins B and C (SP-B and SP-C, respectively) in BALF from seven patients with *ABCA3* mutations and from a control without ILD. Dashed arrows indicate proSP-C (15 and 25 kDa) and proSP-B (15 and 25 kDa). Plain arrows indicate mature SP-C (3.7 kDa) and SP-B (8 kDa).

Similar to the WT protein, *ABCA3* mutants co-localized with LysoTracker staining and partially with ERTracker staining (Fig. 5). *ABCA3* might co-localize with ER during their folding. However, no accumulation in the ER was observed. The same results were obtained with transiently transfected cells (data not shown). Thus, p.D253H and p.T1173R mutants were not associated with a localization defect. *ABCA3* expression was studied on protein extracts from A549-transfected cells. In WT, p.T1173R and p.D253H cells, anti-GFP antibody revealed two bands of 180 kDa (150 kDa *ABCA3* + 30 kDa GFP) and 220 kDa (190 + 30 kDa GFP), respectively (Fig. 6). As previously suggested, these two bands might reflect two processing forms (15–18).

Lamellar bodies in *ABCA3* WT, p.D253H and p.T1173R cells. Lamellar bodies were not observed in A549 cells (Fig. 7). As shown previously with HEK293 cells (15), transfection of *ABCA3* in A549 cells is sufficient to induce lamellar body formation (Fig. 7). The p.D253H mutation induced abnormal lamellar bodies with electron-dense inclusions (Fig. 7, dashed arrows), consistent with the results of the patient's lung biopsy (Fig. 3C). In cells transfected with the p.T1173R mutation, abnormal lamellar bodies are the most frequently observed (irregularly arranged, phospholipid lamellae but eccentrically packed), even if some appeared almost normal.

Cytokine production by *ABCA3* WT, p.D253H and p.T1173R cells. We next investigated whether *ABCA3* mutants could induce inflammation. IL-8 was chosen as a well-known marker in inflammatory chronic lung disease. Using quantitative PCR (qPCR), we found that IL-8 mRNA levels were increased in p.T1173R cells (Fig. 8A). A significant, faint increase in IL-8 mRNA expression was also observed between WT and p.D253H cells. *ABCA3* mRNA levels were similar in WT and mutated cells (data not shown), indicating that the increased IL-8 mRNA level in p.T1173R cells was not due to a transfection issue. At the protein level, ELISA results confirmed that A549 cells expressing the p.T1173R mutant produced more IL-8 than did WT cells (Fig. 8A). In contrast, IL-8 production by p.D253H cells was similar to that of WT cells (Fig. 8B). Finally, TGF- β and MCP-1 secretions were similar between WT and transfected cells (data not shown).

IL-8 production is controlled chiefly by MAPK and NF- κ B signaling (19). To determine whether these signaling pathways were involved in the observed IL-8 overproduction by p.T1173R cells, we used specific inhibitors. Treatment of cells with inhibitors of MAPK (p38, JNK, ERK1/2) and NF- κ B showed that IL-8 production (in WT and mutant cells) was mainly ERK1/2 dependent. The lack of involvement of NF- κ B was confirmed by measuring NF- κ B promoter activity as done previously (20) (data not shown). However, with

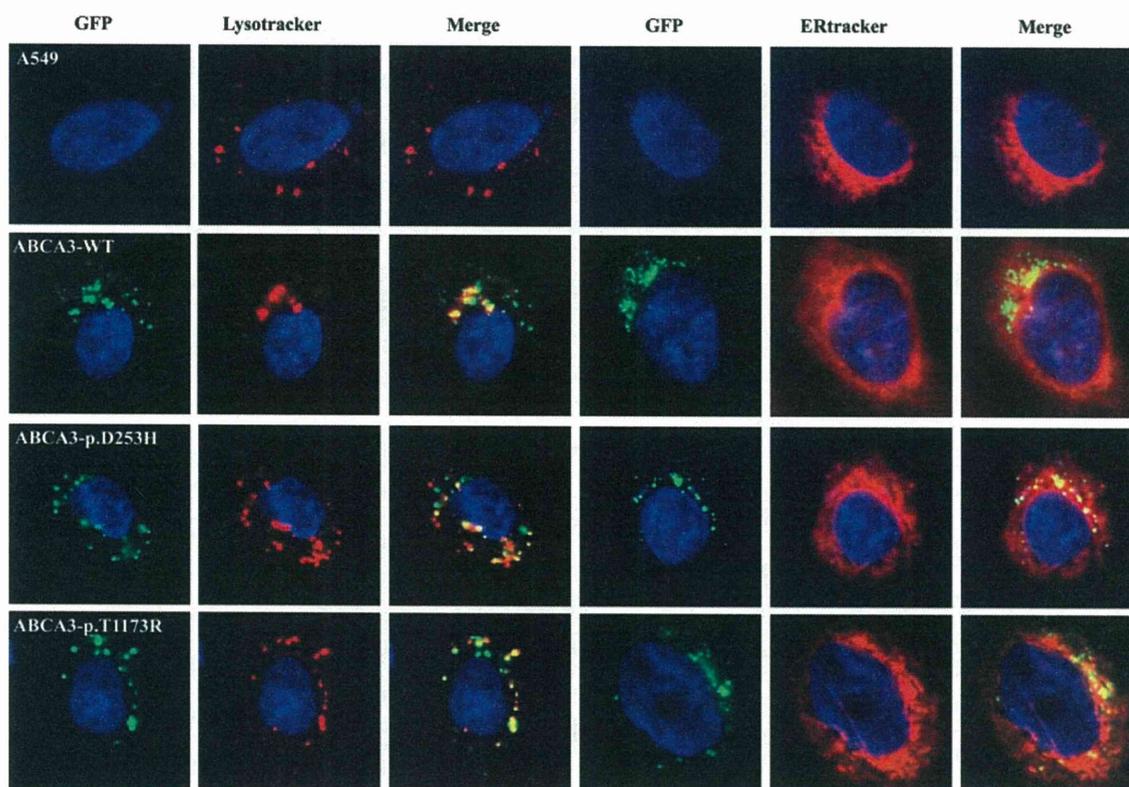


Figure 5. Intracellular localization of wild-type ABCA3 and of the p.D253H and p.T1173R mutants. A549 cells either non-transfected or transfected with mock vector, wild-type protein ABCA3-WT (A) or mutated proteins p.D253H (B) and p.T1173R (C) were analyzed using confocal microscopy. Lysotracker and ERtracker were used to stain lysosomes and the endoplasmic reticulum, respectively.

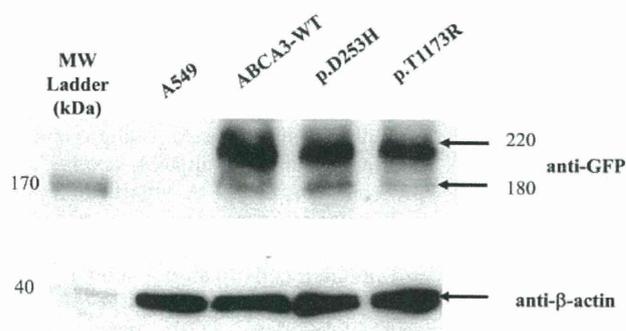


Figure 6. Western blot analysis of ABCA3 in cells transiently transfected with ABCA3-WT or the p.D253H or p.T1173R mutation. ABCA3 expression was detected using anti-GFP antibody (top panel). Equal loading was verified using anti- β actin antibody (bottom panel).

ERK1/2 inhibitor treatment, IL-8 production by p.T1173R cells remained increased compared with WT and p.D253H cells. These results suggest that, even if ERK1/2 signaling is involved in IL-8 production, another signaling pathway may be involved in the increased IL-8 production detected in p.T1173R cells.

Western blot and relative quantification of ERK1/2 phosphorylation confirmed that the observed IL-8 overproduction in p.T1173R cells was independent of ERK1/2 signaling.

Finally, caspase 3/7 activity was similar in WT and mutant cells, indicating that these mutations did not induce apoptosis.

DISCUSSION

We identified 15 (13 novel) *ABCA3* mutations in 47 children (32%) who had NRD and/or ILD and no *SFTP*B or *SFTP*C mutations (5). None of these mutations was found in either the public polymorphism database or our controls. The amino acids affected by the mutations were conserved in mammalian *ABCA3* sequences. All 10 patients with *ABCA3* (21%) mutations had severe respiratory symptoms and abnormal chest imaging findings. Ninety percent of patients harboring *ABCA3* mutations had NRD. Finally, parents heterozygous for the p.R1583W, p.S128Rfs, p.R1521W or p.R208V mutations were not affected. In three patients, a mutation was found in a single allele but the clinical phenotype (NRD) may support the existence of a second mutation (in introns, deletions and so on) not detected by our sequencing method. Haploinsufficiency has been suggested as a mechanism leading to clinical phenotype emergence in patients with only one mutated allele (10,21). Similarly, *Abca3*^{+/-} mice, despite normal respiratory function, had fewer lamellar bodies and altered surfactant lipid synthesis compared with wild-type mice, suggesting susceptibility to NRD or ILD (22). However, even if the observed clinical phenotypes are

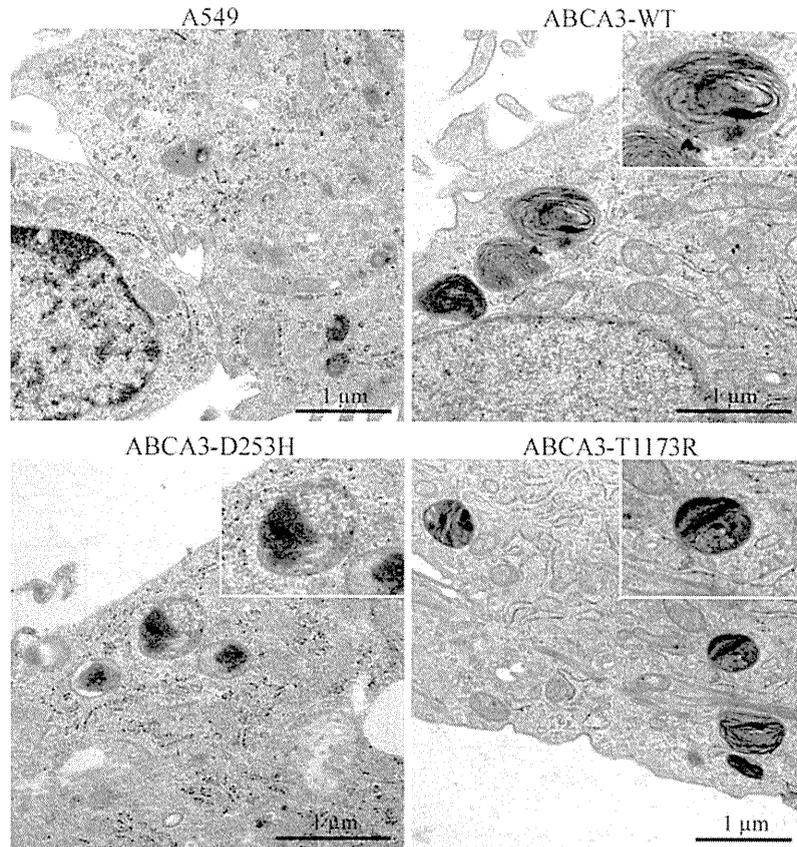


Figure 7. Lamellar body analysis. A549 cells transfected with mock vector (pEGFP-N1), ABCA3-WT (A), or mutated ABCA3-D253H (B) or ABCA3-T1173R-GFP were analyzed using electron microscopy.

compatible with *ABCA3* deficiency, we cannot conclude that *ABCA3* heterozygosis is responsible for this phenotype.

As previously described (1,11,13), some patients with *ABCA3* mutations had a less severe phenotype than that usually associated with *ABCA3* mutations (10). These variations in the clinical and radiological features may be related to the nature of the mutation (16). Previous studies showed that *ABCA3* mutations led to abnormal processing and/or trafficking of the ABCA3 protein (15), alterations in ABCA3 protein functions such as ATPase activity (16), or impaired lipid transport (23). Interactions with variants in other genes and/or with external factors such as viral infections may also influence the observed phenotype (24).

ABCA3 deficiency in full-term patients with NRD was shown previously to be associated with abnormal processing of SP-B and SP-C with an accumulation of precursors of SP-B and absence of mature SP-C (11). In our patients with *ABCA3* deficiency, we observed that SP-C and SP-B expression levels varied considerably across patients. Indeed, less severe patients had decreased amounts of SP-B and SP-C, whereas patient who died of respiratory failure showed normal expression of both proteins. This discrepancy may be explained by our technique of western blot, which is performed on lyophilized supernatant and improved the level of surfactant protein detection. It may also be explained by the

function of ABCA3, which is critical for the proper formation of lamellar bodies and surfactant function but is not responsible for SP-B or SP-C synthesis. Hence, the pattern of SPs may be independent of the clinical status. To confirm the hypothesis, it would have been interesting to compare the patterns of siblings harboring the p.D253H mutation. Unfortunately, BALF from this patient was not available. Finally, as reported previously for *SFTPC* mutations (25), the presence or absence of SP-B and SP-C might be neither sensitive nor specific for *ABCA3*-related diseases.

Electron microscopy examination of a lung biopsy specimen is the reference standard for evaluating lamellar body characteristics and for providing a preliminary diagnosis prior to *ABCA3* gene analysis. Indeed, abnormal lamellar bodies with electron-dense inclusions have been described in association with *ABCA3* mutations in previous studies (10,21,26). However, electron microscopy cannot be performed routinely. The presence of abnormal lamellar bodies in the patient harboring the p.D253H mutation supports a pathogenic effect of this mutation. These abnormalities were also observed *in vitro* in p.D253H- and p.T1173R-transfected cells, suggesting that *ABCA3* abnormalities may consistently induce abnormal lamellar bodies. However, since we do not have the corresponding biopsy from the patient harboring the p.T1173R, we cannot draw a firm conclusion on this point.

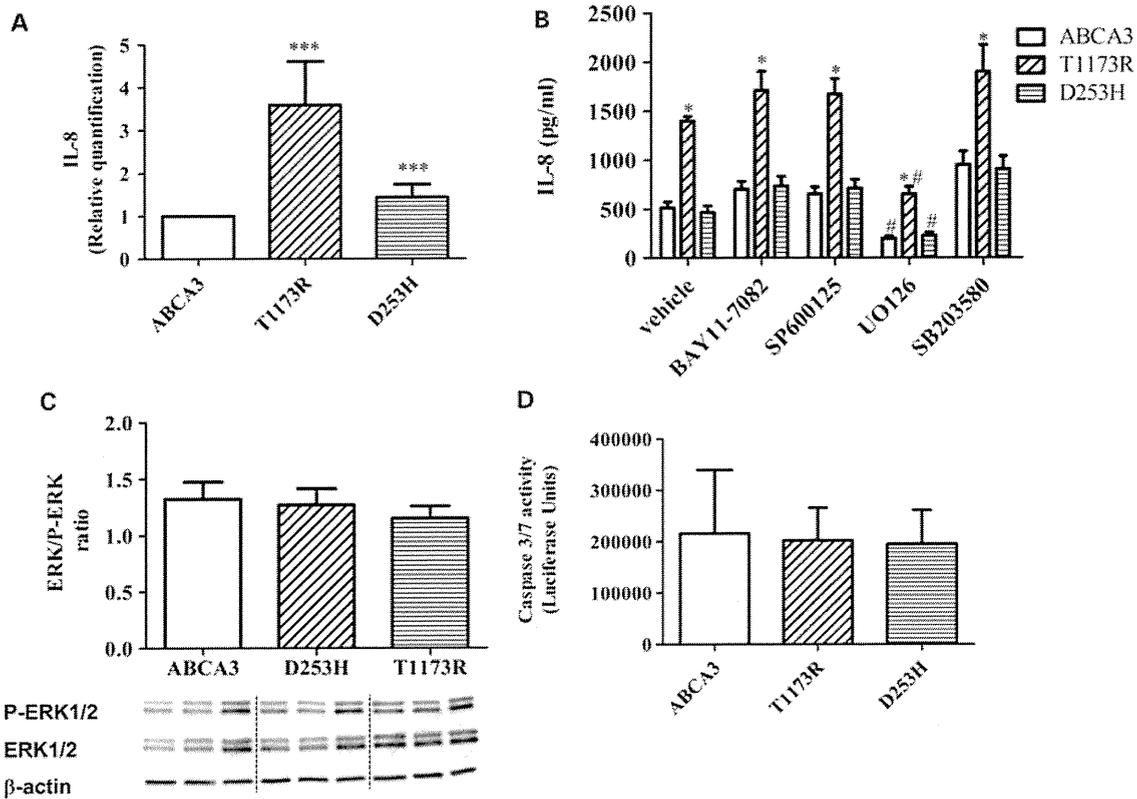


Figure 8. (A) IL-8 mRNA relative quantification was performed using qPCR. Results are representative of the mean \pm SD of three experiments performed in triplicate. *** $P \leq 0.001$. (B) Role for MAPK and NF- κ B-dependent signaling in IL-8 secretion by ABCA3-WT, D253H and T1173R cells. Cells were treated with 10 μ M inhibitors of ERK1/2 (U0126), p38 (SB203580), JNK (SP600125) or NF- κ B (BAY11-7082) for 24 h; * $P \leq 0.05$: ABCA3 versus T1173R; # $P \leq 0.05$: vehicle versus U0126. Supernatants were tested for IL-8 by ELISA. Data are means \pm SD of three experiments performed in triplicate. (C) ERK1/2 phosphorylation measurement. Data are means \pm SD of four experiments performed in triplicate. Results are expressed as P-ERK/ERK ratio (top panel). Western blot analysis of phospho-ERK1/2, total ERK1/2 and β -actin (bottom panel). (D) Caspase 3/7 activity. Data are means \pm SD of three experiments performed in triplicate.

We performed *in vitro* experiments to elucidate the pathophysiological effects of two mutations associated with progression towards ILD, p.D253H and T1173R. These mutations did not alter the localization or maturation of the protein. Past functional studies on other *ABCA3* mutations showed localization/folding defects or functional defects (15,16,18,21,23,27). As pointed out recently, the effect of *ABCA3* mutations on lung epithelial cells depends on the *ABCA3* protein defects (18). We found that the functional abnormalities differed between the two mutations. The p.D253H and p.T1173R mutations induced abnormal lamellar bodies. *ABCA3* being a major transporter of phosphatidylcholine and phosphatidylglycerol into lamellar bodies, the lamellar body alterations suggest abnormalities in phospholipid trafficking that need to be characterized. The recent proteomic characterization of lamellar bodies may help to achieve this challenging objective (28). The p.T1173R mutation was also associated with increased production of IL-8, a well-known chemotactic molecule for neutrophils. Interestingly, increased IL-8 production was also detected in cells expressing *SFTPC* (Δ exon 4 and the common p.I73T) mutations (29). In contrast, we do not find differences in TGF- β production, a cytokine that has been extensively studied in adults with idiopathic pulmonary fibrosis (30). Also, no differences in MCP-1

production were observed between WT and mutated cells. MCP-1 has been shown previously to contribute to the pathogenesis of pediatric ILD (31). However, in this study, none of the children had familial ILD, and surfactant genetic screening was not done, thus we do not know whether MCP-1 production is related to surfactant-associated disorders. In fact, surfactant genetic disorders are a subclass of pediatric ILD (2), which include various clinical phenotypes associated with specific clinical and biological features. Altered intercellular signaling was also shown recently in cells expressing *SFTPC* variants. CXCR1 and CCR2 expression by lymphocytes and neutrophils is probably dependent on an unidentified soluble mediator secreted by p.I73T cells (32). These studies, combined with our data, suggest that inflammatory pathways are involved in genetic surfactant disorders. However, better characterization of these pathways is required if specific treatments are to be sought.

In conclusion, although rare, *ABCA3* deficiency should be considered in full-term newborns with severe respiratory distress and in older patients with ILD. Since *ABCA3* mutations lead to distinct functional defects, functional analysis of each *ABCA3* mutation is necessary to identify specific molecular targets that could be modulated or corrected by therapeutic agents.

MATERIALS AND METHODS

Patients

Through a national program on rare lung diseases, which has been described elsewhere (5), we recruited 121 pediatric patients with diffuse lung disease, over a 5-year period (2002–2007). Among the 121 patients with diffuse lung disease, 86 had respiratory distress, 59 presenting with neonatal onset (NRD) and 18 patients died. *ABCA3* genetic screening was performed in 47 children with severe respiratory distress or familial history compatible with autosomal recessive inheritance. Patients with presence of *SFTPC* or *SFTPB* mutations or insufficient information from medical records were excluded.

For each patient, we retrieved the following information from the medical records: family history, clinical presentation, findings by radiography and HRCT of the chest and lung biopsy findings (including those obtained by electron microscopy). The control for the BALF study was one child with uveitis who underwent bronchoscopy because of suspected sarcoidosis. Bronchoscopy showed no evidence of sarcoidosis or ILD. The control population for the genetic tests consisted of 23 individuals of European descent who had no history of lung disease. The protocol was accepted by the appropriate Committee for the Protection of Individuals in Biochemical Research, as required by French legislation. Written informed consent was obtained from the patients or their next of kin before study inclusion (5).

Genetic analysis

Genomic DNA was extracted from blood samples using an automated BioRobot EZ1 workstation (Qiagen, Hilden, Germany). Parental DNA was sequenced when samples were available. *ABCA3* primers were designed to amplify the 30 coding exons and their respective splice junctions (10). Primers were purchased from Sigma-Aldrich (Lyon, France) and Taq polymerase from Applied Biosystems (Foster City, CA, USA). Sequencing reactions were performed as described previously (5). Identified mutations were verified on two PCR products. Nucleotide numbering reflected *ABCA3* cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001089.2. The reference sequence NP_001080.2 of *ABCA3* protein was used for amino acid numbering.

Histological examination of lung tissue

Lung tissue obtained by surgical biopsy was examined by light microscopy using a standard hematoxylin and eosin staining protocol. Electron microscopy was conducted using standard protocols.

Collection of BALF

We retrospectively analyzed BALF from seven children with *ABCA3* mutations. Fiber-optic bronchoscopy with bronchoalveolar lavage was performed under sedation, as previously described (33).

ABCA3 vectors

The pEGFPN1-*ABCA3* plasmid, called *ABCA3*-WT hereafter, was obtained as described previously (16). Mutagenesis was induced using PCR-based site-directed mutagenesis (Quik-Change Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Mutagenesis primers (Sigma) were as follows: *D253H-For*-5'-ACCCGCCGTTTCATCGCACACCCCTTCC-3', *D253H-Rev*-5'-GGAAGGGGTGTGCGATGAACGGCGGGT-3'; *T1173R-For*-5'-ACGTGCGTGCCTTCAGGCGGGACG-3', and *T1173R-Rev*-5'-CGTCCCCTGAAGGCACGCACGT-3'. Mutagenesis was confirmed by sequencing.

Cell culture and transfection

A549 cells were cultured as described previously (34). Cells (1×10^6) were transfected with 1 μ g of *ABCA3*-WT, *ABCA3*-D253H or *ABCA3*-T1173R plasmid using a nucleofector device (Lonza, Cologne, Germany) as recommended by the manufacturer. For stable transfection, GFP-positive cells were selected using a FACSaria cell sorter (BD, Le Pont-De-Claix, France) and plated with 0.5 mg/ml of Geneticin (Invitrogen, Paisley, UK). Three weeks after selection, stably transfected cells were examined by immunofluorescence and maintained with 0.3 mg/ml of Geneticin. Experiments with transiently transfected cells (Lipofectamine, Invitrogen) were performed 24 h post-transfection. Analysis of NF- κ B activation was done with NF- κ B luciferase plasmid (20).

Fluorescence microscopy

Cells transfected transiently or stably with A549 were plated in 35 mm Petri dishes (iBidi, Martinsried, Germany). Living cells were stained with either Lysotracker red (lysosome probe) or ERtracker red (endoplasmic reticulum probe) (Invitrogen, Paisley, UK). DAPI (Sigma-Aldrich, Lyon, France) was used to stain the nucleus. Fluorescence microscopy was achieved using a Zeiss Axiovert 200 microscope (Zeiss, Le Pecq, France).

Cytokine/ERK ELISA and caspase 3/7

Cells stably transfected with A549 (1×10^5) were seeded in 96-well plates (TPP, Trasadingen, Switzerland). After 24 h, the cells were incubated with vehicle (DMSO) or 10 μ M inhibitors of ERK1/2 (U0126), p38 (SB203580), JNK (SP600125) (Sigma-Aldrich) or NF- κ B (BAY11-7082) (Calbiochem, San Diego, CA, USA). Human IL-8, MCP-1 and TGF- β concentrations in cell culture supernatants were determined 24 h later using the DuoSet enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). Relative ERK1/2 phosphorylation was measured using a cell-based ERK1/2 ELISA kit (RayBiotech, Norcross, GA, USA) following the manufacturer's instructions. Caspase 3/7 activity (Promega, Madison, WI, USA) was measured as recommended by the manufacturer.