Unexplained neonatal respiratory distress due to congenital surfactant deficiency

Marco Somaschini[1], Lawrence M. Nogee[2], Isabella Sassi[3], Olivier Danhaive[4], Silvia Presi[5], Renata Boldrini[6], Cristina Montrasio[7], Maurizio Ferrari[5,7,8], Susan E. Wert[9] , Paola Carrera[5,7]

[1] *Division of Neonatology, Ospedale Bolognini, Seriate, Italy*

[2] *Department of Pediatrics, Division of Neonatology, Johns Hopkins University School of Medicine, Baltimore, Md, USA*

[3]*Pathology Unit, San Raffaele Scientific Institute, Milano, Italy*

[4]*Division of Neonatology and Laboratory of Neonatal Biology, Bambino Gesù Children's Hospital, Roma, Italy*

[5]*Clinical Molecular Biology Laboratory, Diagnostica e Ricerca San Raffaele - Laboraf, Milano, Italy*

[6]*Electron Microscopy Unit, Division of Pathology, Bambino Gesù Children's Hospital, Roma, Italy*

[7]*San Raffaele Scientific Institute, Unit of Genomics for Diagnostics of Genetic Diseases, Milano, Italy*

[8] *Vita e Salute University , Milano, Italy*

[9] *Department of Pediatrics, Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center and theUniversity of Cincinnati College of Medicine,Cincinnati, Ohio, USA*

Key words:

Surfactant protein B
ABCA3
Respiratory distress
Surfactant
Newborn

Corresponding Author and reprint requests:

Dr. Marco Somaschini
Divisione di Neonatologia e Patologia Neonatale
Ospedale Bolognini
Via Paderno 21
24068 Seriate (BG)
tel +39 035 3063307
fax +39 035 3063554
e-mail marco.somaschini@bolognini.bg.it

ABSTRACT

Genetic abnormalities of pulmonary surfactant were identified by DNA sequence analysis in 14 (12 full-term, two preterm) of 17 newborn infants with fatal respiratory distress of unknown etiology. ABCA3 deficiency (N = 12) was a more frequent cause of this phenotype than SP-B deficiency (N = 2).

INTRODUCTION

Inherited deficiency of pulmonary surfactant has been recognized as an emerging cause of acute respiratory distress in neonates and infants.[1,2] Surfactant protein B (SP-B) deficiency was the first recognized inherited disorder of pulmonary surfactant.[3] The disease is due to mutations in the gene encoding SP-B (*SFTPB*) and is transmitted in an autosomal recessive fashion. Mutations in the gene encoding ATP-binding cassette transporter A3 (*ABCA3),* which is critical for proper formation of lamellar bodies, have been identified recently and cause recessive lung disease with a phenotype similar to that of SP-B deficiency with acute respiratory distress.[4] Mutations in the gene encoding surfactant protein C (*SFTPC*) may also cause neonatal lung disease, although more typically result in interstitial lung disease in older children and adults.[5] We analysed *SFTPB, SFTPC* and *ABCA3* DNA sequences for mutations in a group of newborn infants without a specific cause for their respiratory distress in order to determine their relative contributions to this phenotype.

METHODS

Patients

From February 1997 until December 2005, DNA samples from 15 full-term and 2 preterm newborn infants with unexplained respiratory distress were analysed for mutations in *SFTPB, SFTPC* and *ABCA3* after informed consent was obtained from their parents under protocols approved by the institutional review boards of both the referring institutions and where the analyses were performed. Infants had onset of respiratory distress within 24 hours after birth with progressive worsening, requiring ventilatory support. Chest radiographs showed diffuse opacities and granularity, similar to that of premature infants with RDS. All failed to respond to various modalities of ventilatory support, including conventional mechanical ventilation, high frequency oscillatory ventilation (HFOV), inhaled nitric oxide, and exogenous surfactant administration, and died within 7 months. In 7 infants a family history of fatal neonatal lung disease was present. Parental DNA samples were

also analyzed from all infants in whom genetic variants were identified, except for patient 8 whose parents' samples were not obtained.

Molecular analysis.

Direct sequencing of *SFPTB*, *SFPTC*, and *ABCA3* was performed on genomic DNA extracted from 2-3 ml of peripheral blood by phenol-chlorophorm standard technique or by the Quiagen Biorobot EZ1 extractor following the manufacturer's instructions. The analysed regions included the coding exons and intron-exon junctions. The promoter regions were not analysed. Primer sequences are available on-line as supplementary material and PCR conditions are available on request. PCR reactions were purified with the PCR 96 Cleanup Montage kit (Millipore) on a Automated Liquid Handling System (Perkin Elmer) equipped with a vacuum device (Millipore). Dye-terminator cycle sequencing reactions (Dyenamics ET Terminator, Amersham Biosciences) were performed on the purified PCR products with the same primers used for PCR and following the manufacturer's instructions. All sequencing reactions were purified with the G-50 Multiscreen TM-HV plates (Millipore) and then loaded on an ABI Prism 3100 DNA Analyzer (Applied Biosystems). Called sequences were assembled and compared to the sequences in genomic databases (ABCA3 NCBI-NM001089; SFTPC NCBI-J03890; SFTPB NCBI-M24461) with the GeneCodes software, version 3.1.1. (Gene Codes Co.). The frequency of new missense variants was examined in at least 100 control DNA samples (200 chromosomes) obtained from unrelated individuals from the general Italian population with the same ethnic background as patients.

Light microscopy and immunohistochemical staining for SP-B

Histopathologic examination of the lung tissue was performed after routine fixation and staining with hematoxilyn and eosin, as well as with periodic acid-Schiff (PAS) reagent. Formalin-fixed lung tissue was stained for the surfactant proteins using polyclonal antisera directed against SP-B, pro-SP-B, and pro SP-C (Chemicon, USA), as previously described. [6, 7]

Electron microscopy in ABCA3 deficient patients

For ultrastructural studies, open chest lung biopsy fragments were either fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), post-fixed in 1% osmium tetroxide (OsO_4), stained en block with 2% uranyl acetate as previously described [4,8], then dehydrated up to absolute ethanol and embedded in EMbed-812, or fixed in Karnowsky's fixative, post-fixed in osmium tetroxide and processed into epoxy resin. Ultrathin sections were obtained from several blocks, stained with lead citrate and uranyl acetate, and observed with a Zeiss EM 10C transmission electron microscope operated at 60 kV. Control lung tissue was obtained from a term newborn who underwent pulmonary lobectomy for congenital cystic adenoid malformation. The human lung tissue analysed in this study was obtained after parental informed consent from patients' biopsy material sampled for clinical purposes.

RESULTS

Clinical characterisitics and findings of specific gene mutations are summarized in the table. In 2 siblings (patients 4 and 5) molecular analysis showed the presence of the 121ins2 mutation on one allele of the *SFTPB* gene. Direct sequencing of exon 4 indicated a frameshift mutation on the other allele, a single base deletion in a sequence of 5 cytosines spanning codons 120 to 122 of the SP-B cDNA; the first of these two children was the subject of a previous report. [9] Light microscopy findings in the lung tissue of these 2 infants showed an accumulation of granular eosinophilic material that filled the distal airspaces and stained positively with periodic acid-Schiff reagent. Immunohistochemical staining demonstrated the absence of staining for both proSP-B and mature SP-B, even with the aid of antigen retrieval techniques to enhance sensitivity of the immunostaining.

Beginning in 2004, *ABCA3* analysis was performed prospectively and 6 infants were found to have mutations in this gene. Infants enrolled prior to 2004 who were negative for *SFTPB* mutations were

studied retrospectively and 6 of them had *ABCA3* mutations identified. Patients 6 and 15 were siblings.

Fourteen different variants in the *ABCA3* gene were identified in 12 patients. 11 were single base substitutions and 3 were small deletions. Transmission of identified variants from the parents was confirmed in all patients from whom parental DNA samples were obtained and was consistent with mutations being on different alleles. All the missense substitutions except S341N involved highly conserved codons consistent with their being pathogenic mutations. Conservation of codons between orthologues (canis, mus, rattus, fugu, drosophila) was evaluated on amino acid sequences aligned using computer software (data not shown).[10] Two of the missense mutations (R155Q, R208W) were found on more than one affected chromosome in unrelated patients. Patients 10, 11 and 14 were homozygous for the mutant allele, although a history of consanguinity was obtained only for patient 11. The newly identified variants in the symptomatic patients were not detected in 200 chromosomes examined from the general population. As there is 99% likelihood of detecting a variant with a minor allele frequency of 1% in 192 unrelated chromosomes, this indicates that they are not common polymorphisms.[11]

Light microscopy in 6 patients showed type II cell hyperplasia, fibroblast proliferation in interstitial spaces and alveolar lumens containing variable amounts of amorphous, PAS-positive material and clusters of macrophages, features interpreted as consistent with desquamative interstitial pneumonia (Table). Immunohistochememical analysis showed staining for proSP-B, mature SP-B and proSP-C in type II cells, as well as some SP-B staining in intra-alveolar material.

Electron micrographs from 4 infants revealed abnormal lamellar bodies in their type II cells, consisting of clusters of small lamellar bodies, filled with thin, dense and poorly organized concentric membranes with one or two peripheral electron dense cores (Figure). The number of such organelles varied from patient to patient and from cell to cell within the same patient's sections. Patient 10, who was homozygous for a frameshift mutation which would be predicted to preclude ABCA3 expression completely, showed the most markedly decreased number of lamellar

bodies. Patients 9, 12, and 17, who were compound heterozygotes for different missense mutations, also had some abnormal lamellar bodies, along with some normal appearing lamellar bodies. These findings suggest that some *ABCA3* variants may retain some function, and that genotype may predict the severity of the phenotype with respect to lamellar body morphology.

DISCUSSION

A genetic mechanism causing congenital surfactant deficiency was identified in 14 of 17 (82%) newborn infants with unexplained fatal respiratory distress, indicating the importance of genetic studies in infants with this phenotype. ABCA3 deficiency was more frequent than SP-B deficiency and no infants with *SFTPC* mutations were identified. Both SP-B deficient infants were compound heterozygotes for frameshift mutations, including the most common mutation causing SP-B deficiency (121ins2)**;** and immunohistochemical analysis confirmed the absence of SP-B in lung tissue.[7, 9,12,13]

Fourteen different *ABCA3* mutations were identified, including twelve not previously reported, consistent with the allelic heterogeneity observed in previous studies for this disorder.[4,14]

Evaluation of infants with unexplained respiratory failure will thus require a protocol allowing for detection of novel genetic variants, such as direct sequencing or denaturing high performance liquid chromatography combined with direct sequencing. The sensitivity of such a protocol might be increased by also analysing the promoter and other untranslated regions as additional studies identify key regulatory sequences needed for proper ABCA3 expression.

ABCA3 deficiency is the most recently recognized inherited disorder of pulmonary surfactant. ABCA transporters are a family of transmembrane proteins that translocate a variety of substrates across extra and intra-cellular membranes with an ATP-dependent mechanism. Genetic variation in these genes is the cause of or a contributor to a wide variety of human inherited disorders, including cystic fibrosis, neurological diseases, cholesterol and bile transport defects, and anemia. [15] ABCA3 has been shown to be involved in transmembrane transport of endogenous lipids into lamellar

bodies and is critical to the proper formation of lamellar bodies and surfactant function. [16] Ultrastructural findings of abnormal lamellar body formation also supported the diagnosis of ABCA3 deficiency in four infants.[4,8,13]

The majority of reported infants affected by SP-B or ABCA3 deficiency survived only after lung transplantation.[17] However, partial and transient SP-B deficiency compatible with prolonged survival has been recognized.[18,19] Similarly, prolonged survival with chronic interstitial lung disease has been observed in patients with *ABCA3* mutations, suggesting that different mutations may be associated with milder phenotypes and development of chronic lung disease [4, 20] Genetic studies for *ABCA3* are thus warranted in patients with this phenotype.

No mutations in *SFTPB, SFTPC, or ABCA3* were found in three infants, all of whom died within 3 days. Mutations may have gone undetected, other as yet unidentified genetic mechanisms might have been responsible for their fatal lung disease, or they could have suffered from unrelated conditions.

The lung disease associated with mutations in the ABCA3 gene is inherited in an autosomal recessive manner. However, in three patients (7, 8, 13) we found only one *ABCA3* mutation. A second mutation may have been missed in these patients. Large rearrangements or deletions would not have been identified with our protocol, nor would mutations in regions of the ABCA3 gene not analyzed, such as the promoter region. It is also possible that the mutations in these children were a coincidental finding and unrelated to their fatal lung disease.

Although they are rare, SP-B and ABCA3 deficiencies have an emerging role in the etiology of neonatal respiratory distress of unknown cause. There are approximately 600,000 live births per year in Italy and patients in this study were recruited from 11 different medical centers, which represent about 10% of Italian NICUs. Our study was not population based, and we do not know whether all neonates in these centers with refractory respiratory failure were referred for study, precluding accurate estimates of the incidence of these disorders. *ABCA3* mutations were more

prevalent than *SFTPB* mutations. All patients were of Italian origin and whether these findings apply to other populations with different ethnic backgrounds is unknown.

Molecular analysis is a noninvasive test and should be considered in newborn infants who develop progressive respiratory distress without any specific cause of their lung disease. A history of unexplained respiratory failure in a previous child should further strenghten the suspicion. Two infants in this study were born prematurely, including one with a birth weight of < 2 kg, indicating that a diagnosis of genetic surfactant deficiency should also be considered in premature infants with refractory respiratory failure.

Lung tissue analysis may also aid in the diagnosis. Features of what is termed desquamative interstitial pneumonia (DIP) and alveolar proteinosis are highly suggestive of congenital surfactant deficiency in neonates. Specific immunohistochemistry techniques may be helpful. Reduced or absent staining for SP-B is usually observed in patients with *SFTPB* mutations, although may also be observed in association with ABCA3 mutations.[14,20] Extracelluar staining for proSP-C has been consistently observed in patients with *SFTPB* mutations, but not yet reported in association with *ABCA3* mutations.[7,12,14] Characteristic abnormalities of lamellar bodies within alveolar type II cells may be observed by electron microscopy in both ABCA3 and SP-B deficiencies, and ultrastructural evaluation should be part of the tissue assessment in biopsies or autopsies of infants with this phenotype. While treatment is primarily supportive, early identification is important to establish appropriate management and evaluation of treatment options, and to offer genetic counselling and prenatal diagnosis.[21]

Further studies are required to evaluate the incidence of these diseases, to establish genotype-phenotype correlations, to identify both environmental and genetic factors that modify the course of the disease, and to identify other mechanisms responsible for this phenotype.

REFERENCES

1. Nogee LM. Genetic mechanisms of surfactant deficiency. *Biol Neonate* 2004;85:314-8
2. Whitsett JA, Wert SE, Trapnell BC. Genetic disorders influencing lung formation and function at birth. *Hum Mol Genet* 2004;13:207-15
3. Nogee LM, deMello DE, Dehner LP, Colten HR. Deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. N Engl J Med 1993; 328: 406.
4. Shulenin S, Nogee LM, Annilo T, Wert SE, Whitsett JA, Dean M. *ABCA3* gene mutations in newborns with fatal surfactant deficiency. *N Engl J Med* 2004;350:1296-303.
5. Nogee LM, Dunbar AE, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. N Engl J Med 2001;344:573-9
6. Williams GD, Christodoulou J, Stack J,Tobias V, Wert SE, Murrell MJ, Nogee LM. Surfactant protein B deficiency: clinical, histological and molecular evaluation. *J Paediatr Child Healt*h 1999;35:214-20
7. Nogee, LM, Wert, S, Proffit, S, Hull, W, and Whitsett, JA. Allelic Heterogeneity in surfactant protein B deficiency. *Am J Respir Crit Care Med* 2000; 161:937-981.
8. Edwards, V, Cutz, E, Viero, S, Moore, AM, and Nogee, L. Ultrastructure of Lamellar Bodies in Congenital Surfactant Deficiency. *Ultrastructural Pathology* 29: 503-509, 2005.
9. Somaschini M, Wert SE, Mangili G, Colombo A, Nogee LM. Hereditary surfactant protein B deficiency resulting from a novel mutation. *Intensive Care Med* 2000;26:97-100.
10. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by qualità analysis tools. *Nucleic Acid Res* 1997; 25:4876-82.
11. Kruglyak L, Nickerson D.A.. Variation is the spice of life. *Nature Genet*, 2001; 27: 234-236
12. Nogee LM. Alterations in SP-B and SP-C expression in neonatal lung disease. *Annu Rev Physiol* 2004;66:601-23
13. Tredano M, Griese M, de Blic J, Lorant T, Houdayer C, Schumacher S et al. Analysis of 40 sporadic or familial neonatal and pediatric cases with severe unexplained respiratory distress: relationship to *SFTPB*. *Am J Med Genet* 2003;119A:324-39
14. Brasch F, Schimanski S, Muehlfeld C, Barlage S, Langmann T, Aslandis C, et al. Alteration of the pulmonary surfactant system in full-term infants with hereditary ABCA3 deficiency. *Am J Respir Crit Care Med* 2005;172:1026-31

15. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome research* 2001;11:1156-66
16. Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y et al. ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells. *FEBS* 2001;508:221-5
17. Hamvas A, Nogee LM, Mallory GB, Spray TL, Huddleston CB, August A, et al. Lung transplantation for treatment of infants with surfactant protein B deficiency. *J Pediatr* 1997; 130: 231-9.
18. Klein JM, Thompson MW, Snyder JM, George TN, Whitsett JA, Bell EF, et al. Transient surfactant protein B deficiency in a term infant with severe respiratory failure. *J Pediatr* 1998; 132: 244-8.
19. Dunbar AE, Wert SE, Ikegami M, Whitsett JA, Hamvas A, White FV, et al. Prolonged survival in hereditary surfactant protein B (SP-B) deficiency associated with a novel splicing mutation. Pediatr Res 2000;48:275-82
20. Bullard JE, Wert SE, Whitsett JA, Dean M, Nogee LM. ABCA3 mutations associated with pediatric interstitial lung disease. Am J Respir Crit Care Med 2005;172:1026-31
21. Stuhrmann M, Bohnhorst B, Peters V, Bohle RM, Poets CF, Scmidtke J. Prenatal diagnosis of congenital alveolar proteinosis (surfactant protein B deficiency). *Prenat Diagn* 1998; 18: 953-5

Figure 1

A: transmission electron microscopy of lung section in a patient with ABCA3 deficiency shows an alveolus lined exclusively with cuboidal, type II epithelial cells (Pn), the lumen of which is occupied by a cluster of macrophages (Mph). Clusters of small, dense organelles, irregularly distribited in Pn, correspond to abnormal lamellar bodies (arrows) (original magnification: 4,000x). B: higher magnification of lamellar bodies in the same patient (10,000x). C: lamellar bodies in a control subject (10,000x).

List of abbreviations:

SP-B: surfactant protein B

proSP-B: SP-B proprotein

SFTPB: Gene encoding surfactant protein B.

SP-C: surfactant protein C

proSP-C: SP-C proprotein

SFTPC: Gene encoding surfactant protein C.

ABCA3: Adenosine Triphosphate-binding cassette protein, member A3

ABCA3: gene encoding ABCA3

DIP: Desquamative interstitial pneumonia

PCR: polymerase chain reaction

RDS: respiratory distress syndrome

HFOV: high frequency oscillatory ventilation

PAS: periodic acid-Schiff reagent