Genetic surfactant dysfunction in newborn infants and children with acute and chronic lung disease

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Abstract

Mutations in genes encoding surfactant protein B (SP-B), ATP-binding cassette transporter A3 (ABCA3) and surfactant protein C (SP-C) can result in neonatal and pediatric lung disease. We retrospectively reviewed 391 molecular analyses of genes encoding SP-B (SFTPB), SP-C (SFTPC) and ABCA3 (ABCA3) performed in our laboratory from 2000 to 2015 in term and preterm newborn infants with severe respiratory distress syndrome (RDS), infants and children with interstitial lung disease (ILD), chorionic villi for prenatal diagnosis, parents and siblings of affected infants. Direct sequencing of SFTPB, SFTPC and ABCA3 was performed on genomic DNA extracted from peripheral blood. Histopathologic, immunohistochemical and ultrastructural analyses were performed when lung tissue was available. Genetic variants in SFTPB, SFTPC and ABCA3 were identified in 71 of 181 (39%) term and preterm newborn infants tested for severe and unexplained RDS and in 38 of 74 (51%) infants and children with ILD. A higher mortality rate was recorded among term newborn infants with homozygous or compound heterozygous mutations in SFTPB and ABCA3. Light microscopy and immunohistochemical analysis of the lung tissue were performed in 11 infants and electron microscopy in 8. Prenatal diagnosis was performed in 8 women with a previous child who died because of ABCA3 deficiency; 2 fetuses affected, 5 carriers and 1 normal were identified. Surfactant dysfunction was identified in a significant number of newborn infants with severe unexplained respiratory failure and children with ILD, indicating the importance of genetic studies in infants and children with this phenotype. While actual 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.
Introduction

Deficiency of pulmonary surfactant due to immaturity is the most important cause of respiratory distress syndrome (RDS) in preterm newborn infants. Severe and progressive RDS in term newborn infants may result from mutations in genes encoding surfactant proteins, which are important for surfactant metabolism and function [1, 2]. Initially reported as causes of severe RDS in neonates, it has become increasingly evident that these genetic disorders are an important cause of interstitial lung disease (ILD) in children and adults [3-5]. Surfactant protein B (SP-B) deficiency was the first recognized inherited disorder of pulmonary surfactant and has an autosomal recessive inheritance [6]. Mutations in the gene encoding ATP-binding cassette transporter A3 (ABCA3), which is critical for proper formation of lamellar bodies, could cause lung disease with a phenotype similar to that of SP-B deficiency with acute respiratory distress in neonates and ILD in children [7]. Mutations in the gene encoding surfactant protein C (SP-C) have an autosomal dominant pattern of inheritance and may also cause neonatal lung disease, although more typically result in interstitial and chronic lung disease in older children and adults [8]. The main characteristics of genetic surfactant dysfunction are reported in Tab. 1.

Patients and methods

We retrospectively reviewed 391 molecular analyses of genes encoding SP-B (SFTPB), SP-C (SFTPC) and ABCA3 (ABCA3) performed in our laboratory from 2000 to 2015 in term and preterm newborn infants with severe RDS, infants and children with ILD, chorionic villi for prenatal diagnosis, parents and siblings of affected infants. Unexplained neonatal RDS was the presenting symptom in term newborn infants. We also analyzed preterm newborn infants with gestational age (GA) ≤ 32 weeks presenting particularly severe RDS. Clinical presentation of infants and children with ILD consisted of chronic cough, frequent pulmonary infections, failure to thrive and oxygen dependency.

Molecular analysis

Molecular analyses have been performed on genomic DNA extracted from peripheral blood

<table>
<thead>
<tr>
<th>Protein (gene)</th>
<th>Inheritance</th>
<th>Mechanism</th>
<th>Clinical presentation/diagnosis</th>
<th>Onset/disease</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactant protein B (SFTPB)</td>
<td>AR</td>
<td>Loss of function</td>
<td>RDS, hypoxic respiratory failure. Radiographic opacification typical of RDS</td>
<td>Newborn infants during the first hours or days</td>
<td>Fatal</td>
</tr>
<tr>
<td>Surfactant protein C (SFTPC)</td>
<td>AD</td>
<td>Gain of toxic function or dominant negative</td>
<td>Variable respiratory symptoms in neonatal and pediatric age. Radiographic diffuse alveolar damage, interstitial feature of inflammation typical of ILD</td>
<td>Newborns, infants and children</td>
<td>Variable</td>
</tr>
<tr>
<td>Adenosine triphosphate (ATP)-binding cassette transporter A3 (ABCA3)</td>
<td>AR</td>
<td>Loss of function</td>
<td>RDS, hypoxic respiratory failure in newborns. ILD in infants and children. Radiographic finding of alveolar disease and atelectasia</td>
<td>Newborns, infants and children</td>
<td>Severe, fatal, variable</td>
</tr>
</tbody>
</table>

AR: autosomic recessive; AD: autosomic dominant; RDS: respiratory distress syndrome; ILD: interstitial lung disease.

**Table 1. Genes involved in neonatal and paediatric surfactant dysfunction.**
leukocytes by Maxwell 16 extractor (Promega Co., WI, USA) or by conventional phenol-chloroform method. All exonic and flanking regions of SFTPB, SFTPC and ABCA3 genes were amplified by polymerase chain reaction (PCR) and directly sequenced with the Sanger method on a 3730 Applied Biosystems sequencer. The PCR purification and dye removal steps were carried out using a Biomek FX Laboratory Automation Workstation (Beckman Coulter) with AmPure and CleanSeq kit (Agencourt), respectively.

Using Sequencer software (GeneCodes), sequences were compared with each gene reference sequence:
• SPB: ENSG00000168878 and ENST00000409383;
• SPC: ENSG00000168484 and ENST00000318561;
• ABCA3: ENSG00000167972 and ENST00000301732.

All identified variants were described in accordance with “Human Genome Variation Society” (HGVS) nomenclature. HGMD mutation database and literature were used to assess the pathogenicity of the variants.

To forecast the effect of unidentified variants we used Poliphen, Mutation Tasting and Sift software for functional prediction and Fruitfly for splice site prediction.

Since 2016 a new sequencing technology has become available in our lab on a MiSEQ (Illumina) sequencer; some data on surfactant genes obtained with next-generation sequencing technology are yet to be published [9].

**Lung pathology**

Light microscopy and immunohistochemical analysis was performed in deparaffinized formalin-fixed, paraffin-embedded (FFPE) tissue sections stained with hematoxilyn and eosin, as well as with periodic acid-Schiff (PAS), or processed for immunohistochemistry using polyclonal antisera directed against SP-B, pro-SP-B, pro-SP-C (Chemicon, USA) and ABCA3 (Atlas Antibodies, Sweden) as previously described [10].

For ultrastructural studies, open chest lung biopsy fragments were either fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) or fixed in Karnowsky’s fixative, post-fixed in 1% osmium tetroxide (OsO4), stained en bloc with 2% uranyl acetate as previously described [11], then dehydrated up to absolute ethanol and embedded in EMbed-812. Ultrathin sections were obtained from several blocks, stained with lead citrate and uranyl acetate, and observed with a Zeiss EM 10C transmission electron microscope operating at 60 kV.

**Results**

Genetic variants in SFTPB, SFTPC, ABCA3 were identified in 71 of 181 (39%) newborn infants tested for severe and unexplained RDS and in 38 of 74 (51%) infants and children with ILD. Genes affected are reported in Tab. 2. Newborn infants with RDS were divided into three groups according to gestational age: preterm (≤ 32 w), late preterm (33 w to 36 w), and term (≥ 37 w). Consanguinity was present in 15 cases and familiarity for pulmonary diseases in 30 cases. A higher mortality rate (20 newborn infants) was observed among term newborn infants with gestational age ≥ 37 w with homozygous or compound heterozygous variants in SFTPB and in ABCA3. Identified variants were missense, nonsense, synonymous, intronic or splicing, deletions and insertions. Variants with minor allele frequency (MAF) > 0.01 were considered polymorphisms. SFTPB variants were identified in 5 term newborn infants. In 2 of them with compound heterozygous mutations, light microscopy findings in the lung tissue

<table>
<thead>
<tr>
<th>Clinical presentation</th>
<th>Tested</th>
<th>Affected</th>
<th>ABCA3 variants</th>
<th>SFTPB variants</th>
<th>SFTPC variants</th>
<th>Infants with variants in more than 1 gene</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDS, GA ≤ 32 w</td>
<td>68</td>
<td>24</td>
<td>17</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>RDS, GA 33-36 w</td>
<td>31</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>RDS, GA ≥ 37 w</td>
<td>82</td>
<td>35</td>
<td>32</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>ILD</td>
<td>74</td>
<td>38</td>
<td>25</td>
<td>3</td>
<td>15</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
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RDS: respiratory distress syndrome; GA: gestational age; ILD: interstitial lung disease.

Table 2. Number of tested infants with different clinical presentation and number of infants with surfactant genes dysfunction.
showed an accumulation of granular proteinaceous eosinophilic and PAS positive material that filled the distal airspaces (Fig. 1). Immunohistochemical staining demonstrated the absence of staining for both pro-SP-B and mature SP-B. Heterozygous variants of SFTPB were identified in 7 prematures with GA ≤ 32 w and in 3 children with ILD.

Beginning in 2004, ABCA3 analysis was performed prospectively and 32 term newborns, 12 late preterm, 17 preterm and 25 children with ILD were found to have mutations in this gene. Autopsy light microscopy in 9 newborn infants with ABCA3 dysfunction showed type II cell hyperplasia, fibroblast proliferation in interstitial spaces and alveolar lumens containing variable amounts of amorphous, PAS-positive material, features interpreted as consistent with desquamative interstitial pneumonia. Immunohistochemical analysis showed staining for mature SP-B and pro-SP-C and absence of staining for ABCA3 in type II cells (Fig. 2). Electron microscopy from 8 newborn infants with ABCA3 dysfunction 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 (Fig. 3). The number of such organelles varied from patient to patient and from cell to cell within the same patient’s sections. Homozygous for frameshift mutations infants, which would be predicted to preclude ABCA3 expression completely, showed the most markedly decreased number of lamellar bodies, whereas infants with compound heterozygous for different missense mutations or premature infants with heterozygous mutations had some abnormal lamellar bodies, along with some normal appearing lamellar bodies, suggesting that some ABCA3 variants may retain some function. Prenatal diagnosis on chorionic villi between the 10th and the 12th week of gestation was performed in 8 women with a previous child who died because of ABCA3 deficiency. Two foetuses were found to be affected, 5 were carrier and 1 was normal. In the 2 cases where foetuses were affected, pregnancy was terminated. Genetic variants in SFTPC were identified in 3 newborn infants (1 preterm and 2 late preterm) and in 15 infants and children with ILD. In 10 infants genetic variants were identified.

Figure 1. Lung biopsy specimen of a newborn infant with SP-B deficiency. Histopathologic examination of the lung tissue shows intraalveolar accumulation of proteinaceous material (haematoxilin-eosin stain, original magnification x 25).
Figure 2. Representative immunostaining performed on serial slides of a lung biopsy from newborn patient ABCA3 null. Pro-pulmonary surfactant-associated protein C (A), pulmonary surfactant-associated protein B (B), and ATP-binding cassette sub-family A member 3 (C). Positive immunoreactions are observed as a brown precipitate. Intense immunohistochemical expression of SP-C to confirm hyperplasia of type II pneumocytes. No notable staining for ABCA3 was observed in any cell. BAR 50 µm.

Figure 3. Ultrastructural features of disorders caused by mutations in ABCA3 gene: lamellar bodies with electron-dense aggregates in the form of “fried egg” with an asymmetric core (arrows). BAR 2 µm.
in more than one gene (in ABCA3 and SFTPC in 6 infants, in ABCA3 and SFTP in 4).

Discussion

Surfactant dysfunction was identified in a significant number of newborn infants with severe, unexplained respiratory failure and children with ILD, indicating the importance of genetic studies in infants and children with this phenotype. Although rare, surfactant dysfunction is associated with considerable morbidity and mortality. Our study is not population based because this population is highly selected on severe lung disease, precluding estimates of the incidence of these disorders. ABCA3 dysfunction was the most frequently identified defect either in newborns with RDS or in children with ILD. In 15 cases, parents’ consanguinity was present. In newborn infants clinical symptoms of severe respiratory distress are evident in the first hours or days with diffuse lung disease that clinically and radiographically resembles RDS of premature infants, in the absence of other risk factors such as infection. The lung disease is progressive and unresponsive to maximal medical treatment and may result in death within a few weeks or months after birth [12]. However, partial and transient SP-B deficiency compatible with prolonged survival has been recognized [13]. Animal experiments using genetically engineered mice indicate that a level of SP-B production of 20-30% is needed for normal lung function [14]. Thus, infants who are heterozygous for one loss-of-function mutation could be at risk of respiratory failure if other environmental factors such as prematurity further reduce SP-B expression on the other allele. We identified SFTP, SFTPC and ABCA3 single mutations in 24 of 68 tested premature newborn infants with GA ≤ 32 w with a particularly severe course of RDS. Premature newborn infants with a single mutation in SFTP or ABCA3 could have reduced production or impaired function of the protein that, in conjunction with pulmonary immaturity, could increase severity of lung disease [15]. Prolonged survival with chronic ILD has been observed in patients with ABCA3 mutations, suggesting that different types of mutations may be associated with milder phenotypes and development of chronic lung disease [16]. Why some infants do not have respiratory symptoms in the neonatal period but develop symptoms later in infancy or in childhood, is unknown [17]. Frequently ILD in older infants and children is caused by mutations in SFTPC, an autosomal dominant inherited disease; so a mutation in one allele is sufficient to cause disease. A common mutation has been observed in SFTPC (I73T) accounting for 20-30% of SFTPC mutations [18]. In our series we identified 14 variants in SFTPC, of which 8 were I73T. Phenotype regarding severity of disease and age of onset of children with SFTPC mutations is highly variable [3]. In evaluating infants and children who present with symptoms of surfactant dysfunction it is important to consider multiple factors, including family history, age of onset, clinical presentation and characteristics of the course of the disease. Initial evaluation of newborn infants with RDS focuses on the severity and rate of progression of the disease, familiarity for respiratory diseases or early infant deaths. Chest radiography (CXR) and computed tomography (CT) scan are usually the first diagnostic tests performed. Molecular analysis is a non-invasive test and should be considered in newborn infants who develop progressive hypoxic respiratory failure with diffuse lung disease without any other specific cause. A history of unexplained respiratory failure in a previous child should further strengthen the suspicion. If lung disease persists after the first week or is especially severe, genetic testing should be considered promptly because such disease has a poor prognosis and results can influence management choices. Approximately 25% of infants with severe refractory RDS have variations in SFTP, SFTPC, ABCA3 [19]. In prematures, testing for surfactant dysfunction should be performed in infants with unusually severe course of RDS, requiring prolonged ventilator support, showing difficulty of extubation or prolonged oxygen dependency. When onset of lung disease is in the neonatal period ABCA3 and SFTP should be analysed first. If tests for these genes are negative and respiratory symptoms persist, then SFTPC analysis should be performed. In families with identified SFTP or ABCA3 mutations, prenatal diagnosis can be proposed. Prenatal diagnosis allows families with familiarity for surfactant dysfunction to orient in advance therapeutic options [20].

Onset of respiratory failure can manifest after the neonatal age and lung disease may have a slowly progressive course with cough, rapid and/or difficult breathing, exercise intolerance, tachypnea, adventitious sounds, retractions, digital clubbing,
failure to thrive, respiratory failure and hypoxemia. Diffuse abnormalities are present in CXR or CT scan. Such infants can be evaluated in a step-wise fashion, with noninvasive testing initially and then selective invasive techniques. A CT scan may suggest ILD diagnosis and genetic testing or lung biopsy confirms definitive diagnosis. Children who present symptoms of interstitial lung disease after the neonatal period should be considered for ABCA3 and SFTPC analysis first.

Interpretation of results sometimes may be problematic, especially when new variants or some missense unreported mutations are identified; in such cases it is difficult to confirm that they are disease-causing or benign sequence variants without functional activity [21]. Usually, causative mutations mainly occur in exons or immediate intron-exon boundaries of SFTPB or ABCA3; nevertheless, it has been demonstrated that one mutation in non-coding region may result in aberrant ABCA3 mRNA splicing with an altered ABCA3 expression [22]. If genetic testing is equivocal or non-diagnostic, lung tissue analysis (particularly immunohistochemistry and electron microscopy) may be useful in making a diagnosis.

Infants with SP-B deficiency almost always have a poor prognosis, whereas mutations in ABCA3 or SFTP C lead to a more variable disease and can have a more favourable prognosis. No specific therapies have demonstrated their effectiveness in treatment of surfactant dysfunction; therefore management is based upon uncontrolled studies, case series or reports. With rare exceptions, SP-B deficiency remains a fatal disease and lung transplantation is the only therapeutic option. The five year survival rate for infants who have undergone lung transplantation is approximately 50% [23]. Many infants with ABCA3 dysfunction have a similar clinical course and may also be candidates for lung transplantation. However, as some infants survive for years, selection of candidates for lung transplant may be difficult. Supportive and preventive care of infants with ILD include treatment of hypoxemia, nutritional failure and prevention of infections. Many infants require oxygen supplementation at home, some require invasive or noninvasive ventilation. Poor somatic growth requiring nutritional intervention is frequently observed. Infants benefit from pneumococcal vaccine, annual influenza vaccination, palivizumab and routine childhood immunization. As steroids increase ABCA3 expression in vitro and in experimental animals, this may constitute a rationale for their use in ABCA3 deficiency. Pulse dose steroids, hydroxychloroquine and azitromycin have been used, but none have been subjected to controlled trials and reported improvements are anecdotal [19]. The decision whether or not to initiate a trial with immunosuppressive therapy must be made on a case-by-case basis, considering severity of the disease, rate of progression, prognosis without therapy and family preferences; infants on pharmacological therapy should be monitored for side effects. Gene therapy is a promising treatment because genetic surfactant deficiencies are monogenic defects, but it is still experimental [24]. While actual treatment is primarily supportive, early identification is important to establish appropriate management and evaluation of treatment options, and to offer genetic counselling and, where applicable, prenatal diagnosis.

Declaration of interest

The Authors declare that there is no conflict of interest.

References