Fatal neonatal respiratory failure in an infant with congenital hypothyroidism due to haploinsufficiency of the \( \text{NKX2-1} \) gene: alteration of pulmonary surfactant homeostasis

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SUMMARY
Defects of the \( \text{NKX2-1} \) gene, encoding thyroid transcription factor-1, cause brain-thyroid-lung syndrome (MIM 610978), characterised by benign hereditary chorea, congenital hypothyroidism and respiratory disease. The case of a term infant with mild primary congenital hypothyroidism and neonatal persistent respiratory failure with fatal outcome at 10 months of age despite continuous ventilatory support is described. Congenital defects of genes known to disturb surfactant protein and lipid homeostasis (\( \text{SFTPB}, \text{SFTPC}, \text{ABCA3} \)) were excluded. Hypothyroidism prompted sequencing of \( \text{NKX2-1} \), which revealed a heterozygous 29 bp deletion (c.278_306del29) disrupting the affected allele. Analysis of bronchoalveolar lavage fluid demonstrated an abnormally low amount of surfactant protein C (SP-C) in relation to SP-B, and low levels of surfactant phospholipids, indicating disturbance of SP and lipid homeostasis as a consequence of \( \text{NKX2-1} \) haploinsufficiency. \( \text{NKX2-1} \) haploinsufficiency may lead to lethal respiratory failure of the newborn due to disruption of pulmonary surfactant homeostasis. \( \text{NKX2-1} \) gene analysis should be considered when investigating irreversible respiratory insufficiency of the newborn.

INTRODUCTION
Fatal respiratory failure in term newborns has been associated with genetic defects disrupting homeostasis of the pulmonary surfactant system, such as mutations in genes encoding surfactant protein B (SP-B) or SP-C or \( \text{ABCA3} \) encoding the lamellar body-associated ABC transporter A3.3 Thyroid transcription factor-1 (TTF-1), encoded by the gene \( \text{NKX2-1} \), has been established as being crucial for lung development and function. In \( \text{NKX2-1} \) null mutant mice, development of the ventral forebrain, pituitary and thyroid is severely impaired, as is lung branching.4 Heterozygous \( \text{NKX2-1} \) defects have been found in patients with benign hypothyroidism and pulmonary alterations5 or in combination with benign hereditary chorea.6,7 Therefore, the term ‘brain-thyroid-lung syndrome’ has been introduced.8 However, any one or even two of these manifestations may be missing. Of the 46 patients reported to date with clinical and molecular genetic information available, respiratory disease appears to be the most inconstant finding (54% of patients identified).9 In a single patient with hypothyroidism and lethal neonatal respiratory failure, a functionally relevant missense mutation of \( \text{NKX2-1} \) (I207F) was reported recently. However, the absence of mutations in genes previously known to be responsible for fatal neonatal respiratory failure such as \( \text{SFTPB} \) or \( \text{SFTPC} \) encoding the hydrophobic SP-B and SP-C or \( \text{ABCA3} \), was not demonstrated.10 Here we report the fatal outcome of a term male infant with mild hypothyroidism and respiratory failure requiring mechanical ventilatory support shortly after birth. We demonstrate the absence of defects in the \( \text{SFTPB}, \text{SFTPC} \) and \( \text{ABCA3} \) genes. Instead, a heterozygous disrupting mutation in \( \text{NKX2-1} \) was identified. A disturbance of pulmonary surfactant composition affecting proteins and lipids is reported.

SUBJECT AND METHODS
Case report
After an uneventful pregnancy, a male infant was born spontaneously at 41 weeks of gestation to a healthy non-consanguineous Caucasian couple as their first child. Postnatal respiratory adaptation was normal, but tachypnea with mild intercostal inspiratory retractions and flaring nostrils was noted on day 3 of life, which prompted transfer to a newborn intensive care unit. On admission, oxygen saturation showed varying readings from 86% to 97% on room air, while respiratory rate was 100/min. Antibiotic treatment was begun. However, a bacterial, viral or parasitic infection was subsequently ruled out, as was a cardiac defect. Routine screening for metabolic diseases on day 4 of life detected an elevation of thyrotropin (thyroid-stimulating hormone (TSH); 48 mU/l; normal <24), while free thyroxine was normal (FT4; 16.4 pmol/l; normal 11–22.3). Treatment with l-thyroxine was begun for congenital hypothyroidism. Since initial FT4 values were normal, several attempts were made to reduce thyroxine substitution. This repeatedly resulted in a significant increase in TSH values, confirming the diagnosis of mild congenital primary hypothyroidism. The presence of maternal antithyroid antibodies was excluded and normal orthotopic thyroid tissue was demonstrated by ultrasound. Chest radiographs showed diffuse ground glass opacity increasing from days 3 to 13 of life (figure 1A). Oxygen supplementation was initiated. At 14 days, the infant was intubated electively for
both parents using standard procedures. Sequencing of the entire coding regions and exon-flanking intronic sequences of the \(SFTPB\), \(SFTPC\) and \(ABCA3\) genes (including immediate promoter regions of \(SFTPB\) and \(SFTPC\)) was performed as previously reported using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Darmstadt, Germany) and an ABI 310 or ABI 3130xl Genetic Analyzer.

Biochemical analyses

Bronchoalveolar lavages (4×1 ml/kg body weight of normal saline) were centrifuged to remove cells and supernatants were analysed for total protein content with the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, California, USA). Then, 10 μg of total protein were separated under non-reducing conditions on NuPage 10% Bis-Tris gels with the help of a NOVEX X-cell II Mini-Cell system (Novex, San Diego, California, USA). SPs and their proforms were detected with polyclonal rabbit antiserum, as described previously. Lipid classes and molecular species were determined by electrospray ionisation tandem mass spectrometry analysis as described previously. Control bronchoalveolar lavage specimens were obtained under previous protocols. Briefly, the samples were from a newborn aged 4 weeks, two 4- and 14-month-old infants (all three with
the diagnosis of bronchitis and absence of parenchymal lung disease) and a 40-year-old adult (healthy control).

RESULTS

DNA analyses

Since the clinical picture was consistent with a congenital disturbance of the pulmonary surfactant system, the SFTPB and SFTPC genes were initially sequenced. No abnormality was detected in SFTPC, while in SFTPB a frequent polymorphism, a G→A exchange at 384 base pairs upstream of the transcriptional start site, in a TTF-1 binding site was present in the heterozygous state. This polymorphism is associated with reduced transcriptional activity of the SFTPB gene.10 ABCA3 sequencing did not reveal a relevant alteration. Sequencing of the NKX2-1 gene led to the detection of a novel heterozygous 29-base pair deletion (c.278_308del) in exon 2 resulting in a frameshift starting at amino acid position 93 and in a premature stop codon (p.Ala93GlyfsX54) rendering the protein non-functional. This mutation was not detectable in leucocyte DNA from the parents.

Biochemical and lipid analyses

At a protein level, serial analyses of bronchoalveolar lavage specimens from postnatal days 14, 98 and 208 demonstrated a consistent reduction in mature SP-C and some aberrantly processed pro-SP-C, while mature SP-B was present in normal amounts (figure 1C). The percentage of surface-active phospholipids phosphatidylcholine and phosphatidylglycerol was severely reduced, as was dipalmitoyl-phosphatidylcholine, demonstrating perturbed surfactant phospholipids homeostasis (table 1).

DISCUSSION

Although several heterozygous mutations (including null alleles) have been reported, there is only a single report describing fatal neonatal respiratory failure due to a NKX2-1 defect.10 Interestingly, the phenotype with respect to the grade of hypothyroidism at birth and the finding of normal thyroid tissue was identical to ours and not among the most severe thyroidal phenotypes observed in carriers of NKX2-1 mutations. Due to the location of the resulting frameshift on the amino terminal side of the conserved domains (home-domain and ‘NKX2-1-specific domain’), there is no doubt about the disruptive nature of the mutation in our case. As speculated by Maquet and colleagues,10 a missense mutation, in contrast, may potentially lead to a dominant-negative effect, for example, through DNA binding in competition with the wildtype protein, due to its inability to properly transactivate. This pathophysiological mechanism can be excluded here.

From a clinical standpoint, the nature of the lung disease was a failure of the pulmonary surfactant system. While the reduced amount of mature SP-C and abundance of its precursors in bronchoalveolar lavage may indicate a disturbance of pulmonary surfactant homeostasis, it may not be as good an explanation for respiratory failure as the reduction in surface-active phospholipid species observed. While this manuscript was being reviewed, Guillot and colleagues10 demonstrated that mutations in NKX2-1, whether associated with a loss or a gain of function, may lead to severe lung disease in early life. In a patient with a loss-of-function mutation, they found a reduction in mature SP-B and SP-C while precursors were abundant.

TTF-1 is known to regulate SFTPC transcription directly17 or via an interaction with TAZ (transcriptional co-activator with PDZ-binding motifs).18 Since TTF-1 also activates transcription of the ABCA3 gene,19 which encodes a lamellar body membrane protein involved in the import of surfactant phospholipids, predominantly phosphatidylcholine and phosphatidylglycerol, it is conceivable that its downregulation results in abnormal pulmonary surfactant lipid composition as shown here. Furthermore, TTF-1 is known to regulate SFTPB transcription.20 One may speculate that the additional finding of an SFTPB promoter polymorphism known to be associated with decreased transcription15 21 is to blame for the severe phenotype. Analyses of bronchoalveolar lavages, however, did not indicate a lack of SP-B.

The question is open, however, why in some cases of NKX2-1 mutations lung disease is absent, transient or mild, while irreversible lung failure from birth is seen in others. Coexisting alterations in other genes (such as the WWTR1 gene encoding TAZ, or others) may be involved. Since postmortem morphological analyses of the lung were declined by the parents, it cannot be ruled out that the lack of SP-C and surfactant phospholipids was combined with a structural problem in the lung (branching defect) leading to a reduced number of alveoli and consequent impairment of gas exchange as reported by Maquet and colleagues.10

We conclude that irreversible lung failure at birth belongs to the clinical spectrum of heterozygous NKX2-1 gene defects. We show that a NKX2-1 defect disrupts pulmonary surfactant homeostasis at the level of surfactant-associated proteins and phospholipids. When investigating possible causes of this clinical presentation, NKX2-1 defects therefore need to be considered and family history should be examined for symptoms of brain-thyroid-lung syndrome.

Table 1  Mass spectrometry phospholipid analysis of pulmonary surfactant material recovered by bronchoalveolar lavage

<table>
<thead>
<tr>
<th>Phosphatidylcholine (% total PL)</th>
<th>Mean of 4 different controls</th>
<th>Case at 3 different time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine (% total PL)</td>
<td>73.2 ± 5.2</td>
<td>53.5 ± 3.4*</td>
</tr>
<tr>
<td>Dipalmitoyl-PC (PC 32:0) (% of total PC)</td>
<td>50.4 ± 2.5</td>
<td>29.9 ± 3.6*</td>
</tr>
<tr>
<td>Phosphatidylglycerol (% total PL)</td>
<td>1.3 ± 0.3</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>Lyso-PC (% total PL)</td>
<td>9.3 ± 3.0</td>
<td>2.1 ± 1.0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (% total PL)</td>
<td>1.7 ± 0.4</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Phosphatidylserine (% total PL)</td>
<td>4.8 ± 1.7</td>
<td>12.4 ± 1.2</td>
</tr>
<tr>
<td>Phosphatidylserine (% total PL)</td>
<td>4.3 ± 1.9</td>
<td>6.8 ± 0.8</td>
</tr>
</tbody>
</table>

Patient samples were taken on postnatal days 14, 98 and 208, at least 2 weeks after surfactant replacement. PL composition in the patient was stable over time. For comparison, lavage material from controls was analysed in parallel. The relative abundance of the typical surfactant PL classes (PC, PG) and of the molecular species PC 32:0 (in % of total PC) were compared by t test. The surfactant-specific species were significantly reduced in the case (*p < 0.05) such that the relative amounts of membrane PLs (phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and sphingomyelin) were increased. PC, Phosphatidylcholine; PL, phospholipids.
Contributors  BK and MG have contributed equally and are to be regarded co-first authors.

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Arch Dis Child Fetal Neonatal Ed 2011 96: F453-F456 originally published online June 28, 2010
doi: 10.1136/adc.2009.180448

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