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An intronic ABCA3 mutation responsible for respiratory disease

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Abstract

Background—Member A3 of the ATP-Binding Cassette family of transporters (ABCA3) is essential for surfactant metabolism. Nonsense, missense, frameshift and splice-site mutations in the ABCA3 gene (*ABCA3*) have been reported as causes of neonatal respiratory failure (NRF) and interstitial lung disease. We tested the hypothesis that mutations in non-coding regions of *ABCA3* may cause lung disease.

Methods—ABCA3 specific cDNA was generated and sequenced from frozen lung tissue from a child with fatal lung disease with only one identified *ABCA3* mutation. *ABCA3* was sequenced from genomic DNA prepared from blood samples obtained from the proband, parents and other children with NRF.

Results—ABCA3 cDNA from the proband contained an sequences derived from intron 25 that would be predicted to alter the structure and function of the ABCA3 protein. Genomic DNA sequencing revealed a heterozygous C>T transition in intron 25 trans to the known mutation, creating a new donor splice site. Seven additional infants with an ABCA3 deficient phenotype and inconclusive genetic findings had this same variant, which was not found on 2132 control chromosomes.

Conclusion—These findings support that this variant is a disease-causing mutation, which may account for additional cases of ABCA3 deficiency with negative genetic studies.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids and proteins produced by alveolar type II cells (AEC2s) that is essential for lowering surface tension at the air-liquid interface and preventing end-expiratory atelectasis (1, 2). Surfactant proteins (SP-), designated SP-A, SP-

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B, SP-C, and SP-D, have important roles in surfactant function and metabolism, as does member A3 of the ATP Binding Cassette Family (ABCA3) of transporters (3, 4, 5). Mutations in the genes encoding SP-B (*SFTPB*), SP-C (*SFTPC*), and ABCA3 (*ABCA3*), have been associated with surfactant dysfunction and respiratory disease in full-term infants with neonatal respiratory failure, and interstitial lung disease in older children and adults (6, 7, 8, 9). Although the precise incidences of these disorders are unknown, *ABCA3* mutations appear to be the most common cause of inborn errors of surfactant metabolism (1, 2, 3).

ABCA3 is expressed in a number of tissues, but most strongly in the lung, and is intimately involved in surfactant function. The 1704 transmembrane protein is localized to the limiting membrane of the lamellar body (LB), the intracellular storage organelle for surfactant in AEC2s and is thought to facilitate transport of lipids into the LB (1). It is encoded by a single gene containing 33 exons, spanning 80 kb on chromosome 16 (4, 5). Over 100 mutations have been identified with disease inherited in an autosomal recessive manner and mutations on both alleles felt necessary for disease (3).

To date, only mutations located in the exons or the immediate intron-exon boundaries that would alter the *ABCA3* coding sequence have been reported as causes of lung disease. Patients with lung disease consistent with *ABCA3* deficiency, and only one or no disease causing mutations identified have been reported (3, 6, 7). We hypothesized that mutations in non-coding regions resulting in aberrant *ABCA3* mRNA splicing and expression may account for some unidentified, abnormal *ABCA3* alleles. To test this hypothesis we investigated the mechanisms for lung disease in a newborn infant with respiratory failure who had only one known disease-causing *ABCA3* mutation identified.

MATERIALS AND METHODS

CASE HISTORY

The index patient was a full-term male infant who developed hypoxemic respiratory failure and pulmonary hypertension shortly after birth. He was placed on extra-corporeal membrane oxygenation on day of life 11. His chest x-ray demonstrated chronic interstitial changes and migratory atelectasis. He was decannulated at 5 weeks of life and minimal improvement was observed despite maximal support, including 100% inspired oxygen, inhaled nitric oxide, sildenafil, and bosentan. Genetic testing performed through the Johns Hopkins University DNA Diagnostic Lab revealed normal *SFTPB* and *SFTPC* gene sequence, but a heterozygous mutation in *ABCA3* (Nt2068G->A, p.E690K) that had previously been identified as disease-causing (1, 2). He died at 3 months of age of progressive respiratory failure with a superimposed acute pneumothorax, and permission for autopsy was obtained. Lung histology findings included some alveolar spaces that were filled with alveolar macrophages and others that contained granular material that stained positively with Periodic acid-Schiff reagent, alveolar epithelial cell hyperplasia, interstitial widening and fibrosis, as well as pulmonary hypertensive changes with increased smooth muscle in the smaller caliber pulmonary arteries. Electron microscopy demonstrated small, abnormal lamellar-like bodies with tightly packed phospholipid membranes and eccentric electron dense inclusions in the cytoplasm of AEC2s typical of those observed in *ABCA3* deficient infants (6).

Study populations—The protocol was approved by the Johns Hopkins University School of Medicine Institutional Review Board (IRB) and written informed consent was obtained from the parents of all participants. The case patient was enrolled in a prospective study aimed at identifying children with inborn errors of surfactant metabolism, who were referred by their primary physician due to the severity of their lung disease. Criteria for enrollment included: 1.) Near-term or full-term gestation (\geq 36 weeks). 2.) Hypoxemic respiratory failure severe enough to need mechanical ventilation. Children with less severe pulmonary disease treated with only supplemental oxygen or positive pressure were eligible if there was a family history of lung disease. 3) Diffuse parenchymal lung disease on chest radiograph. 4.) No definitive etiology for the child’s lung disease. In addition, older children with a clinical diagnosis of interstitial lung disease (ILD) were enrolled under a separate study arm.

A separate study population, evaluated under a separate IRB approved protocol at Washington University, consisted of newborns who were referred for evaluation for a suspected inborn error of surfactant metabolism due to an extreme respiratory phenotype, defined as needing significant and/or prolonged ventilatory support or death, or who had a family history of respiratory disease.

RNA and DNA Preparation—Lung tissue from the proband was snap frozen in liquid nitrogen at the time of autopsy. Eighty micrograms of frozen lung tissue was homogenized and total RNA extracted and reverse transcribed for ABCA3 cDNA using primers and methods previously described (1). Genomic DNA was prepared from both a blood sample and the lung tissue from the proband, and from saliva samples from his parents using a commercially available kit (Qiagen, Gaithersburg, MD).

Mutational Analysis—The 30 coding exons of the ABCA3 gene were amplified by PCR from genomic DNA and overlapping amplicons spanning the entire ABCA3 cDNA amplified by reverse-transcription PCR using primers and conditions previously described (1, 8). Automated DNA sequencing was performed through the Genetic Resources Core Facility at Johns Hopkins University School of Medicine. DNA sequencing chromatograms were analyzed with the aid of Sequencher software (GeneCodes, Ann Arbor, Michigan) and compared to the reference ABCA3 cDNA sequence (NCBI NM_001089). Specific primers were created to generate amplicons expanding from the previously known mutation (p.E690K at exon 17) to the area of interest in intron 25 . Splice site predictions were obtained by using an online informatics website provided by Neural Network (www.fruitfly.org), in which splicing probability for a given sequence was rated by a score from 0 to 1.

Restriction Digest Analysis—Restriction endonuclease *BanII* was purchased from the New England Biolabs (Beverly, MA) and used with supplied reagents according to the manufacturer’s instructions.

Control Samples—RNA was prepared from twelve infants with fatal lung disease due to known *SFTP*B mutations and was reverse-transcribed as previously described (10) and ABCA3 specific cDNA was generated spanning the region of interest (exons 25 to 26) to

serve as control samples for aberrant ABCA3 splicing. Specific primers were designed to incorporate a part of intron 25, and terminate in exon 24 upstream.

For population-based controls, we resequenced the region of intron 25 containing the IVS25-98T variant in DNA extracted from Guthrie cards obtained from the Missouri Department of Health and Senior Services Newborn Screening program as previously described (11, 12, 13). These samples were obtained from infants of both African (N=195) and European-descent (N=871).

RESULTS

In order to determine whether aberrant RNA splicing occurred in the case subject's lung, overlapping amplicons spanning the entire ABCA3 cDNA were generated. Gel electrophoresis yielded products of the predicted sizes except for the products spanning exons 23 to 27. Two additional bands of higher molecular weight in addition to one of the expected size were observed (Figure 1). Sequence analysis of the cDNA revealed that the higher molecular weight bands contained extra sequence between exons 25 and 26 of 150 and 249 base pairs (bp) in length, respectively. The 249 base insertion was derived from the 3' end of intron 25, and the 150 base insertion was derived from the sequence corresponding to the -249 to the -100 positions from the beginning of exon 26. DNA sequence analysis of the remainder of the cDNA did not deviate from the known reference sequence, with the exception of the G>A substitution at c.2068 corresponding to the missense mutation previously identified in the subject's genomic DNA. Gel electrophoresis of amplicons spanning ABCA3 exons 23 to 27 generated from control samples obtained from patients with severe lung disease (SP-B deficient, N =12) yielded products of the expected size. To determine if a low level of alternative splicing and generation of the 249 bp product occurred in the control samples, antisense primers specific for the retained intronic region paired with an upstream primer in exon 21 were used in RT-PCR, but products of the predicted size were only obtained with RNA from the subject's lung and not from control samples (not shown).

In order to determine whether the aberrantly spliced transcripts were derived from the allele with the c.2068 G>A (p.E690K) mutation, primers were designed to amplify either the transcripts containing the intronic sequence or those that were normally spliced, by using a 3' primer located in the intronic sequence, or one that spanned exons 25 and 26. These primers were each combined with a 5' primer located proximally to c.2068 and RT-PCR was used to generate products with either wild-type or intronic-sequence containing transcripts that spanned the site of the c.2068 G>A mutation (Figure 2). The resulting products were then digested with the restriction endonuclease *Ban II* which would cut if A was present at c.2068 (mutation), but not if G was present at this site (wild-type), as well as cutting at several internal *Ban II* sites. Electrophoretic analysis of the restriction products demonstrated that the relevant *Ban II* restriction fragment derived from the aberrantly spliced transcripts was smaller than that derived from the normally spliced products, indicating the presence of the G allele (wild-type) in those amplicons containing the intronic sequences, and the A allele (p.E690K mutation) in those containing normally splicing. (Figure 3B). Sequence analysis of the RT-PCR amplicons also confirmed these findings (not

shown). These results are consistent with the aberrantly spliced transcripts being derived from the opposite allele of that containing the c.2068G>A mutation.

To determine the origin of the aberrant transcripts, intron 25 was sequenced from the proband's genomic DNA. A heterozygous C>T transition at the -98 position of intron 25 was the only sequence variant identified between exons 25 and 26 (Figure 3). In order to determine splicing probability changes with the newly identified variant, the sequence was evaluated using an online splice-site predicting tool. With the IVS25-98T variant, the tool generated a new splicing donor site with a score of 0.24, in contrast to a score of 0 with the IVS25-98 C wild-type.

Genomic DNA was obtained from both parents and analyzed by DNA sequencing of amplicons spanning the site of each identified mutation to determine whether they were in cis or in trans. The mother was found to be heterozygous GA at position c.2068 (site of the p.E690K mutation), but was homozygous CC at position IVS25-98. The father was heterozygous CT at position IVS25-98, but homozygous GG (wild-type) at c.2068. Thus the proband was a compound heterozygote for the c.2068 A and IVS25-98 T variants.

Having identified a potential second disease-causing *ABCA3* mutation in the proband, we next sought to determine whether this finding was unique to this family, or whether this mutation caused or contributed to disease in other children whose phenotypes were consistent with *ABCA3* deficiency, but whose genetic studies failed to identify two mutations. We first identified ten additional infants previously evaluated under our study who shared characteristics similar to the case patient. Specifically they had pulmonary phenotypes felt consistent with *ABCA3* deficiency based upon their lung histopathology and/or family histories, but had only one *ABCA3* mutation identified that was likely to be disease-causing. Three of these infants were heterozygous for IVS-98T (Table 1). We next evaluated DNA from 250 newborns referred for evaluation of an extreme respiratory phenotype, who had not had any mutations in *ABCA3*, *SFTPB* or *SFTPC* identified. Four apparently unrelated neonates from South America who had fatal neonatal respiratory disease consistent with *ABCA3* deficiency were homozygous for IVS-98T. Finally, IVS-98T was not detected in any control samples (N = 1066) by sequence analysis, indicating that it is not a frequently occurring polymorphism among Missouri infants of European or African-Descent.

DISCUSSION

We identified a novel intronic *ABCA3* mutation in a child whose clinical presentation, lung histology, and electron microscopy findings were all consistent with *ABCA3* deficiency, yet who had only one known disease causing mutation identified. Aberrant RNA splicing was derived from the allele opposite to the known mutation, and the genomic intronic variant was also in trans with the known mutation. The identical genomic variant was found in eight additional unrelated children with the phenotype of *ABCA3* deficiency, but whose genetic studies had failed to identify two mutations to account for disease. This variant was not found in control subjects without lung disease. Collectively, these observations support that the IVS-98T mutation is a disease-causing *ABCA3* mutation, and that it may also account for

respiratory disease in children who cause of lung disease has remained unidentified with negative genetic studies for *ABCA3* mutations in commercial or research laboratories.

While aberrant splicing arose from the allele with IVS-98T, the precise mechanism accounting for the observed aberrant transcripts is unclear. Two aberrant transcripts were identified, both containing retained intronic sequences distal to the identified mutation. The IVS-98T mutation creates a new potential donor splice site, with a probability score measured by a splicing algorithm of 0.24, compared to 0 with the wild-type sequence. This, in conjunction with an existing upstream potential splice acceptor site, with a score of 0.63, could account for the 150 bp insertion. This pseudo-exon is predicted to add an additional 50 amino acids to the protein between the 11th and 12th membrane spanning domains, potentially altering the intracellular routing, stability, or function of the *ABCA3* protein. The second aberrant transcript contained the last 249 bases on intron 25. The reading frame would have resulted in a termination codon after an additional 77 amino acids from exon 25 resulting in a truncated *ABCA3* protein, lacking a membrane spanning domain, the second nucleotide binding domain, and the carboxy-terminal domain, and would likely result in non-functional protein. However, the mechanism to account for this larger aberrant transcript with an additional 249 bases is unclear. As there is a potential splice acceptor site in the intron, it is possible that this represents an alternative splicing variant that is present normally at low levels, and that its relative abundance was increased due to the altered splicing with the mutation. Splicing variants, that can occur at low levels in normal individuals, but are expressed at higher levels in the context of mutations affecting normal gene expression have been observed in other disorders, including in the *SP-B* gene (14).

As opposed to previously identified *ABCA3* mutations, this mutation was not located in an exon or an immediate intron-exon boundary, but represented a relatively deep intronic mutation located almost 100 bases from the beginning of exon 26. Deep intronic mutations resulting in aberrant splicing have been observed in other genetic diseases. In cystic fibrosis (CF), due to mutations in another ABC transporter (*ABCC7*), also known as the CF transmembrane regulator (*CFTR*), patients without identified mutations, yet a clinical CF phenotype, have been well documented(15). Recently, a novel intronic mutation characterized by *CFTR* mRNA was found to cause a splicing variant and induce a pseudo-exon in patients without a previously identified exonic mutation(16), similar to the mechanism in our proband. Splicing anomalies are also described with the 5T mutation in CF, where improper splicing leads to removal of an entire exon (1). Similar to this pathogenesis, the role of the IVS-98T intronic variant in *ABCA3* splicing adds to understanding the mechanism of disease in patients in whom the underlying etiology of neonatal respiratory failure is ambiguous.

Four infants with lethal neonatal respiratory failure from South America were homozygous for *ABCA3* IVS25-98T. These infants were not known to be related and there was no clear history of consanguinity. It is possible that these infants were homozygous due to a mechanism such as isodisomic uniparental disomy, obtaining both copies of chromosome 16 from a single parent, which has been reported for *ABCA3* deficiency (4). Parental samples were not available from these infants, precluding studies to investigate this possibility. Alternatively, the frequency of the IVS25-98T mutation may be increased in this

ethnic population. While the South American population based frequency of IVS25-98T is unknown, the incidence may be frequent enough to result in a significant number of homozygous individuals with lung disease. Additional studies are needed to determine this population-based frequency, and these findings may have important implications for this population.

Our findings further support that not all disease-causing mutations are identified in patients with an ABCA3 deficient phenotype, either with the acute neonatal presentation or with later onset chronic interstitial pneumonitis. Additional studies are needed to identify novel genetic mechanisms of heritable disease and improve the sensitivity of genetic testing. Our findings highlight the importance of obtaining tissue suitable for RNA studies in order to specifically investigate splicing abnormalities. Such tissue would also allow more sensitive expression of ABCA3 mRNA and protein levels. Lung tissue obtained from lung biopsy or autopsy specimens from newborns and older children with respiratory failure of unclear etiology should be frozen in liquid nitrogen (5). While ABCA3 IVS-98T was identified in 3 other infants with one ABCA3 mutation and an ABCA3 deficient phenotype, the mechanism for lung disease in the 7 other infants without a second identified ABCA3 mutation remains unknown.

In summary, an intronic ABCA3 mutation disrupting normal ABCA3 mRNA splicing was identified in multiple unrelated children with lung disease. These findings highlight the potential importance of non-coding variants in causing human disease as well as the limitations to the sensitivity of genetic testing which should be considered when interpreting results of genetic studies from children with suspected inborn errors of surfactant metabolism.

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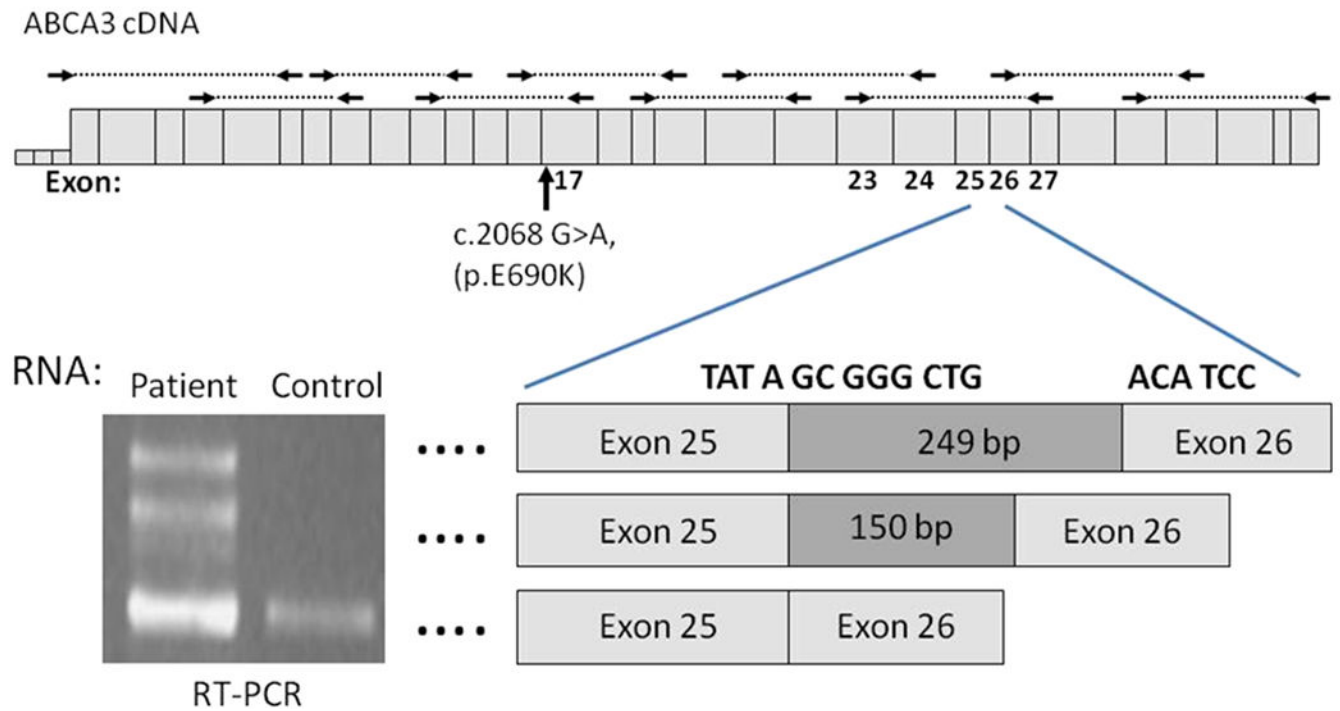


Figure 1. ABCA3 Exons 25–26 Aberrant Splicing

Representative diagram of the ABCA3 cDNA focused on the region of exon 25–26 in the proband. Two additional products can be appreciated migrating at higher molecular weights than observed in the control. Excerpt of DNA sequence shown verifying the presence of the beginning of the intronic sequence corresponding to the additional product.

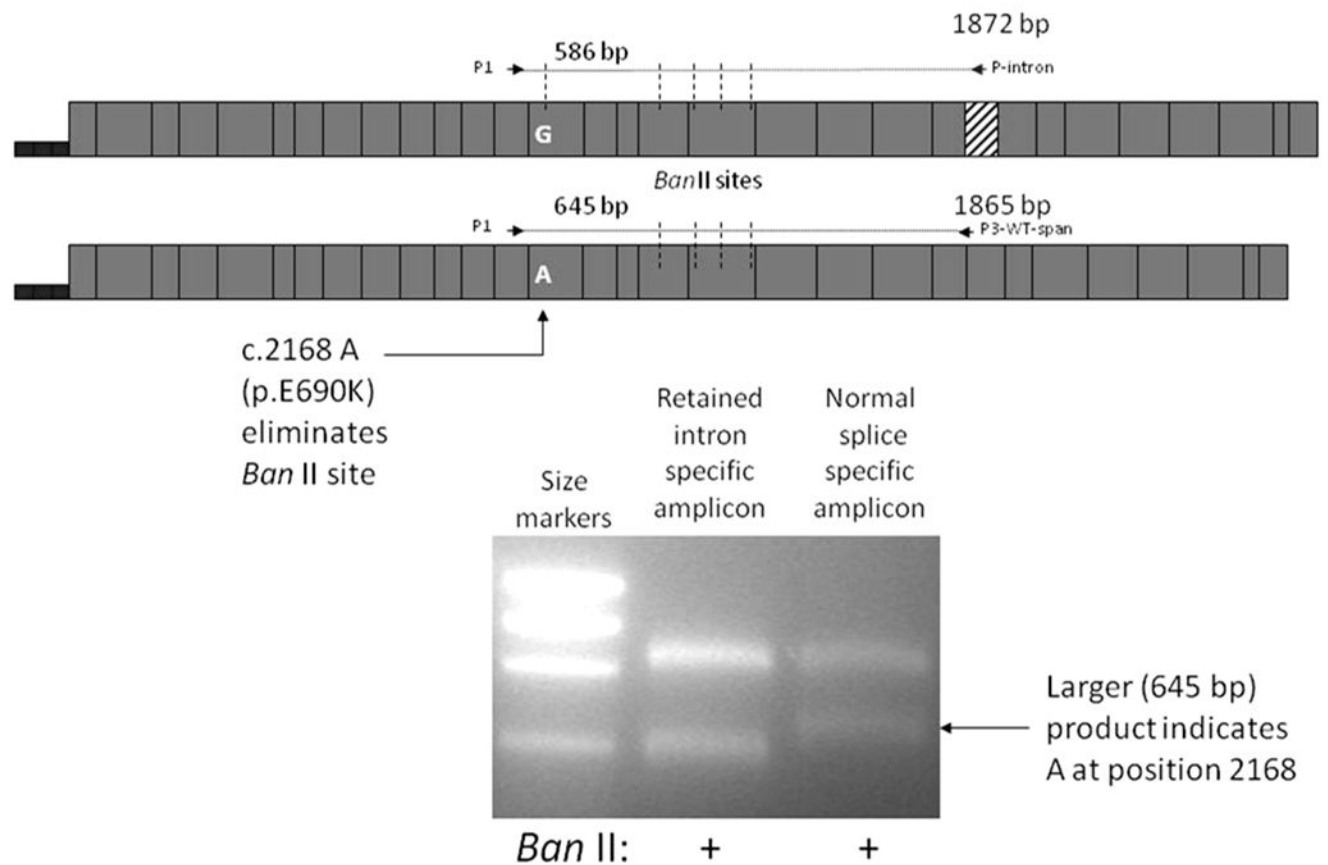


Figure 2. Transcript-specific RT-PCR and *Ban* II RE Digest

ABCA3 cDNA showing allele with inserted intronic sequence corresponding to the non p.E690K allele. Using a *Ban*II restriction digest, the known p.E690K mutation eliminates a restriction site in the normally spliced amplicon, generating a larger product (645bp vs. 586bp), confirming the intronic insertion is in *trans* to the previously identified mutation.

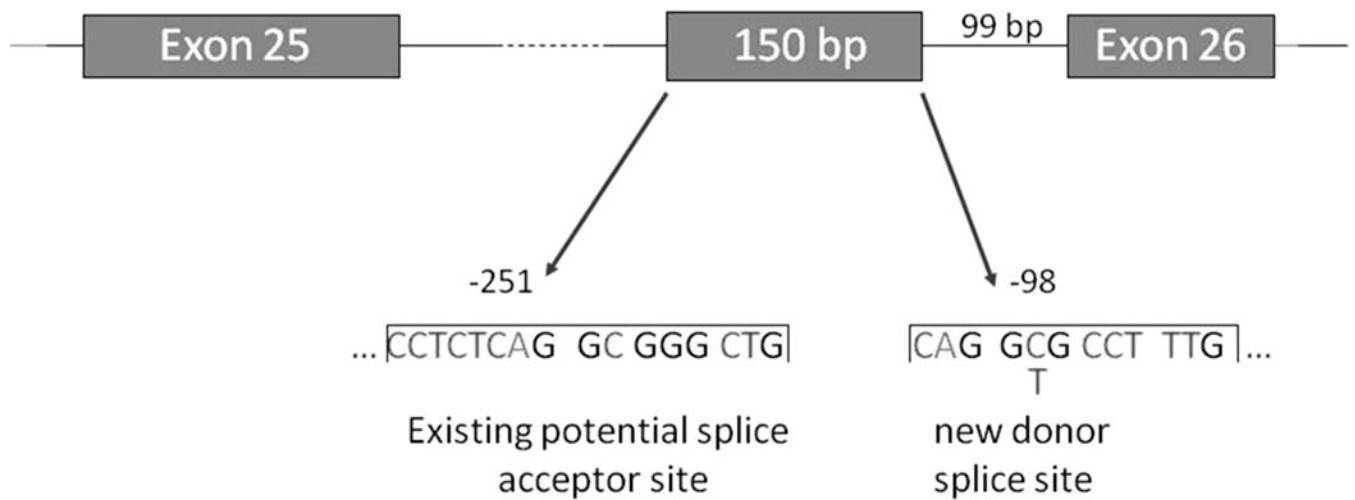


Figure 3. Genomic Sequence Analysis

Genomic sequence with emphasis on the intervening sequence between exons 25 and 26. A C>T transition at the -98 position of IVS25 is present, creating a new donor splice site. An existing upstream potential splice acceptor site is present at the -251 position, giving rise to the 150bp product. DNA sequence analysis is shown verifying the heterozygous C>T transition.

Table 1Characteristic of subjects with one *ABCA3* mutation

Patient	Ethnicity	Presentation	Allele 1 Mutation	Allele 2 Mutation	Findings consistent with <i>ABCA3</i> Deficiency	Outcome
A	Caucasian	Newborn, RDS	p.E690K	IVS25-98T	Case patient; Lung histopathology and EM	Died
B	Caucasian	RDS	p.L941P	IVS25-98T	Family history of sibling with fatal RDS	Died
C	Caucasian	8 y/o, ILD	L212M	?	Mutation associated with disease in other patients	Alive with ILD
D	Caucasian	Newborn, RDS	c.4903ins5	?	Family history of 2 siblings with fatal RDS, lung histopathology and EM	Died
E,F	Caucasian	Newborn, RDS	p.E1325K	?		Died
G	Hispanic	2 months, ILD	p.R43C	IVS25-98T	Lung histopathology and EM	Lung transplant
H	Hispanic	Newborn, RDS	p.A1070T	?	Mutation associated with disease in other patients, lung histopathology	Alive with ILD
I	Caucasian	Newborn, RDS	p.R43H	IVS25-98T	Mutation associated with disease in other patients, lung histopathology	Alive with ILD
J	African-American	ILD	p.R280C	?	Mutation associated with disease in other patients, lung histopathology	Alive with ILD
K	Caucasian	ILD	p.N1418S	?	Mutation associated with disease in other patients	Alive with ILD

Infants identified in a prospectively conducted study evaluating children for inherited disorders of surfactant metabolism who were likely *ABCA3* deficient based upon the finding on one disease-causing mutation and additional findings as listed. Three of these infants (B, G, I) were also heterozygous for *ABCA3* IVS25-98C>T in addition to the case patient (A). RDS = Respiratory Distress Syndrome. EM = Electron Microscopy.