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Cryptic intronic *NBAS* variant reveals the genetic basis of recurrent liver failure in a child

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ABSTRACT

Background: In almost half of patients with acute liver failure the cause is unknown, making targeted treatment and decisions about liver transplantation a challenge. Monogenic disorders may contribute to a significant proportion of these undiagnosed patients, and so the incorporation of technologies such as next generation sequencing (NGS) in the clinic could aid in providing a definitive diagnosis. However, this technology may present a major challenge in interpretation of sequence variants, particularly those in non-coding regions.
Results: In this report we describe a case of Infantile liver failure syndrome 2 (ILFS2; MIM 616483) due to novel bi-allelic variants in the *NBAS* gene. A missense variant NM_015909.3(*NBAS*):c.2617C > T, NP_056993.2(NBAS):p.(Arg873Trp) was identified by whole genome sequencing (WGS). By combining WGS and reverse transcription-polymerase chain reaction (RT-PCR) we were able to identify a novel deep intronic variant, NM_015909.3(*NBAS*):c.2423 + 404G > C, leading to the inclusion of a pseudo-exon. This mechanism has not been described previously in this syndrome.
Conclusions: This study highlights the utility of analyzing NGS data in conjunction with investigating complementary DNA (cDNA) using techniques such as RT-PCR for detection of variants that otherwise would be likely to be missed in common NGS bioinformatic analysis pipelines. Combining these approaches, particularly when the phenotype match is strong, could lead to an increase in the diagnostic yield in acute liver failure and thus aid in targeted treatment, accurate genetic counseling and restoration of reproductive confidence.

1. Introduction

Pediatric acute liver failure is a complex clinical disorder in which rapidly progressive severe hepatic dysfunction presents in a child often without pre-existing liver disease. Patients can present with encephalopathy, ascites, seizures, coagulation abnormalities and elevated liver enzymes as a result of many different etiologies, including infections, exposure to toxins, autoimmune disorders, drug exposure, inherited metabolic and other genetic disorders [1]. Establishing a definitive diagnosis is very important as it impacts the management of these patients as well as their eligibility for possible liver transplantation [2].

The underlying cause of acute liver failure has not been determined in up to 49% of affected children [1]. It is likely that monogenic disorders could explain at least some of these undiagnosed cases [3]. Examples of monogenic disorders that present with acute liver failure in

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children include galactosemia, tyrosinemia, Niemann-Pick C, Wilson disease, fructose intolerance, glycosylation defects, urea cycle defects, Infantile liver failure syndrome 2 (*NBAS* related) and mitochondrial diseases [1,4]. However, there is a clinical overlap between many of these disorders, making it difficult to clinically establish the diagnosis. The traditional diagnostic approach often comprises prolonged, expensive and intrusive diagnostic processes involving clinical evaluation, urine and blood biochemistry, tissue biopsies for histology and enzymology, followed by molecular genetic testing of candidate genes, which delays targeted treatment and decisions about the appropriateness of transplantation [5,6].

More recently, with the introduction of next generation sequencing (NGS) into healthcare, we have an opportunity to achieve a diagnosis in a more efficient and non-invasive manner. The utility of NGS for the diagnosis of other monogenic disorders has already been clearly established. When implemented early in the diagnostic journey in patients suspected to have a monogenic disorder, NGS can increase the diagnostic yield in a faster and cost-effective manner, as opposed to the traditional clinical and laboratory pathways, with some studies reporting a diagnostic yield of up to 60% using an NGS approach, about double that of the "standard" pathway [7,8].

As these technologies continue to become more accessible it is expected that the use of NGS will be increasingly implemented in the diagnostic approach to pediatric acute liver failure [3,6]. However, one of the main challenges of using NGS in the clinic includes the bioinformatic data analysis and interpretation. Most of the algorithms used to identify pathogenic variants do not prioritize those that are outside the protein-coding regions or exon-intron boundaries. Consequently, variants in deep intronic regions that could potentially cause splicing defects are easily overlooked [9]. To overcome this challenge, the analysis of complementary DNA (cDNA) or RNA can be conducted as an orthogonal strategy to assess the impact of candidate deep intronic variants on splicing [10]. In this paper we highlight the utility of WGS in combination with cDNA studies in a child with Infantile liver failure syndrome 2, in whom a novel disease mechanism for the *NBAS* (Neuroblastoma Amplified Sequence) gene was detected by combining both methodologies to identify a deep intronic variant leading to pseudoexon inclusion, *in trans* with a missense variant.

2. Materials and methods

2.1. Patient clinical summary

This girl was born at 38 weeks *via* emergency lower segment caesarean section for maternal pre-eclampsia to non-consanguineous parents of indigenous Australian background. Her birth weight was approximately 4 kg. She was bottle fed. There was no significant family history. She now has 5 siblings who are well.

Early growth and development were normal and she was fully immunised. From the age of thirteen months she began having episodes of fulminant liver failure, usually triggered by an intercurrent viral infection, resulting in transaminases being in the tens of thousands and liver synthetic function becoming deranged (INR up to 9.1 units; RR 1.0–1.2) as manifested by a severe coagulopathy, and associated with lactic acidosis and often hypoketotic hypoglycaemia. Blood ammonia was sometimes mildly elevated during these episodes (peaked at 340 during first admission and treated with sodium benzoate; RR 10–50 μmol/L). Other admissions had milder elevations up to 115 μmol/L.

On a number of occasions, the liver failure was so severe that she almost came to urgent liver transplant. However, with supportive dextrose-containing IV fluids she made a complete recovery over a period of 1–2 weeks each time. In between episodes, her acute hepatomegaly and liver function returned completely to normal, and blood lactate ranged from normal to being intermittently mildly elevated (1.6–3.4 mmol/L; RR 0.7–2.0 mmol/L). There was an impression that treatment with coenzyme Q at the time of acute liver crises shortened the duration of liver failure. She was consequently commenced on regular coenzyme Q and L-carnitine, with the frequency of episodes of liver failure reducing, although it was unclear whether these medications contributed to that or whether heightened awareness in the parents led to admissions earlier in the course of intercurrent illnesses, which seemed to reduce the severity and duration of liver failure (Fig. S1). Despite these episodes she continued to enjoy normal growth and development.

Formal cardiology assessment was unremarkable. Normal investigations include urine amino and organic acids, and plasma total and acylcarnitines, serum transferrin isoforms, alpha 1 antitrypsin levels (M2M2 phenotype), as well as fibroblast fatty acid oxidation studies. A liver biopsy during an acute episode showed only microvesicular steatosis.

Muscle and liver biopsies taken when well showed mild lipid accumulation but were otherwise unremarkable. Mitochondrial respiratory chain enzyme studies were performed as described previously [11]. The liver respiratory chain enzymology was normal, while the muscle results showed possible complex II + III deficiency (22% of normal relative to protein, 14% relative to citrate synthase and 15% relative to complex II). White blood cell coenzyme Q levels were normal.

Mutation testing for hereditary fructose intolerance was negative. No mtDNA deletions were detected in DNA extracted from liver.

When last reviewed at 9 years of age, her last presentation with acute liver failure was three years earlier. She had subsequently been well. The family actively avoid acetaminophen or any other potential liver toxin. They also try to avoid intercurrent illnesses, as these seemed to precipitate her events. She was otherwise growing and developing normally without any other medical issues.

3. Whole exome and genome sequencing and *in-silico* analyses

Genomic DNA was extracted from blood of the proband and parents and whole exome sequencing (WES) and primary bioinformatics analysis were performed as previously described [12]. WGS and variant calling were performed at the Kinghorn Centre for Clinical Genomics (Garvan Institute, Sydney) as described previously [13]. Variants from the MitoCarta 2.0 [14] and selected liver disease genes *POLG, TRMU, DGUOK, BCS1L, MPV17, RRM2B, TYMP, TP, LARS, CPT2, TWNK, NBAS* were prioritized using Seave [15].

In silico analyses of variants were performed using PolyPhen-2 [16] (http://genetics.bwh.harvard.edu/pph2/), SIFT [17] (http://sift.jcvi.org), CADD [18] (http://cadd.gs.washington.edu), MutationTaster [19] (http://www.mutationtaster.org/), Human Splicing Finder v3.1 [20] (http://www.umd.be/HSF3/), and SROOGLE [21] (http://sroogle.tau.ac.il). To determine allele frequencies the Genome Aggregation Database [22] (gnomAD; http://gnomad.broadinstitute.org) was used. Alignments and conservation analysis were conducted using the UCSC Genome Browser [23] (http://genome.ucsc.edu/).

4. Complementary DNA (cDNA) studies

Proband and control fibroblast cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM). Cultured fibroblasts were grown with and without cycloheximide treatment (100 ng/μl) to inhibit nonsense-mediated decay (NMD) as previously described [24].

RNA was extracted using the miRNeasy Mini kit (QIAGEN) and cDNA was synthesized using the Superscript III kit (ThermoFisher) according to the manufacturer's protocol. cDNA was amplified by polymerase chain reaction (PCR) using 12 sets of primers designed to overlap the 52 exons of the *NBAS* sequence (Table S1) followed by Sanger sequencing.

5. Results

Homozygous and compound heterozygous variants in *NBAS* have been previously associated with ILFS2 and SOPH syndrome (Short stature, optic nerve atrophy, and Pelger-Huet anomaly, MIM 614800), the former having a strong phenotype match with the patient.

However, in the trio WES analysis, only one likely pathogenic variant was prioritized in *NBAS*. This variant, which segregated with the mother, was identified in exon 24. The missense variant NM_015909.3(*NBAS*):c.2617C > T, chr2:g.15557797G > A, NP_056993.2(NBAS):p.(Arg873Trp) is predicted to change an arginine to a tryptophan at amino acid position 873. The arginine at this position is highly conserved (Table S2), and is situated in the secretory pathway Sec39-like domain involved in vesicle transport from the endoplasmic reticulum to the Golgi apparatus [25]. *In silico* tools predict this variant to be disease-causing (Table S3). It has been observed in population databases at a frequency of 0.000008131 (2/245,964) but has not been reported in a homozygous state. It has not been previously reported to cause disease.

Also maternally inherited was a likely benign variant found in exon 38; the missense variant NM_015909.3 (*NBAS*):c.4475A > G, chr2:g.15467981 T > C, NP_056993.2(NBAS):p.(Tyr1492Cys) is predicted to change a tyrosine to a cysteine at amino acid position 1492. The tyrosine at this position is not conserved in mammals and is not situated in a known functional domain. *In silico* tools predict this variant to be tolerated. It has been observed in population databases at a frequency of 0.00000407 (1/245712) but not in a homozygous state. It has also not been previously reported to be associated with disease.

Because of the strong ILSF2 phenotype, we went on to perform trio WGS. As with the WES studies, in the initial analysis of WGS data we found only these two maternally inherited variants in *NBAS*. No candidate variants *in trans* were found in the coding region of the *NBAS* gene and a SNP array failed to detect any copy number variant (CNV) changes in the region (data not shown). Given the strong phenotype match, further analysis of the non-coding regions of *NBAS* and cDNA studies were conducted in order to determine whether a second pathogenic allele had been missed.

After re-analysis of the WGS data, a deep-intronic paternally inherited heterozygous variant was identified within intron 22. The NM_015909.3(*NBAS*):c.2423 + 404G > C, chr2:g.15567431C > G variant is located +404 bp from the canonical splice-site of exon 22. This variant has been observed in population databases at a frequency of 0.00006460 (2/30958) but not in a homozygous state. It has not been previously reported to cause disease. *In silico* tools that combine multiple splicing regulatory element algorithms, Human Splicing Finder HSF v3.1 [20] and SROOGLE [21], suggested that the variant could disrupt a splicing silencer motif (Table S4 and Fig. S2).

Patient and control fibroblasts were grown with or without cycloheximide (which inhibits NMD) to assess the impact of this deep-intronic variant on splicing. As a consequence of the NM_015909.3(*NBAS*):c.2423 + 404G > C variant, a new 131 bp pseudo-exon containing intron 22 sequence was created and inserted into the transcript (chr2:15,567,351-15,567,481) (Fig. 1). The predicted consequence at the protein level is the insertion of 9 additional amino acids and the creation of a premature stop-codon p.(Arg809Phefs*10), with loss of over 1500C-terminal amino acids. The aberrantly spliced transcript was observed at low levels in patient cells in the absence of cycloheximide treatment, suggesting that the transcript undergoes NMD. In keeping with this, the c.2617C > T;p.(Arg873Trp) variant appeared homozygous in the cDNA from patient cells grown without cycloheximide as was the c.4475A > G;p.(Tyr1492Cys) variant. Interrogation of RNA-seq data of 151 control fibroblasts from the Genotype-Tissue Expression (GTEx) project did not show evidence of transcripts containing this pseudo-exon. No other abnormalities were detected by RT-PCR of the proband cDNA.

6. Discussion

The *NBAS* gene contains 52 exons and encodes a 2371 amino acid Neuroblastoma Amplified Sequence protein, which is involved in Golgi-to-endoplasmic reticulum (ER) retrograde transport [26]. It has been suggested that the secretory pathway Sec39-like domain is essential for this function and bi-allelic pathogenic variants in this region are associated with ILFS2 [25]. This disorder is inherited in an autosomal recessive manner and patients often present in infancy with recurrent episodes of acute liver failure associated with fever and with complete recovery between intervals [25]. This fits very closely with the clinical history of the patient described here.

Interestingly, patients with a homozygous missense p.(Arg1914His) variant in the C-terminal domain do not have acute liver failure, but have a different clinical presentation, SOPH syndrome [27]. This same variant has been reported in a compound heterozygous state in patients with all of the SOPH cardinal features including optic atrophy and a predominant skeletal phenotype, however they also presented with elevated liver transaminase levels associated with fever without developing acute liver failure [28,29]. Furthermore, it has been recently noticed that some patients with ILFS2 may have some subtle SOPH manifestations including short stature, and mild dysmorphic and skeletal features [25,27,30].

The patient reported here did not show any of the SOPH manifestations, and given that the allele harboring the deep intronic variant leads to NMD resulting in an 'apparent homozygosity' in cDNA of the p.(Arg873Trp) missense variant within the highly conserved Sec39-like domain, this is entirely in keeping with the ILFS2 presentation in this patient.

Intronic variants causing splicing defects have been reported in numerous diseases that can manifest with acute liver failure (Table 1). However, deep-intronic variants located at least 100 bp from the closest canonical splice-site have only been associated with acute liver failure in ornithine transcarbamoylase deficiency (MIM 311250) and Niemann-Pick type C (MIM 257220) (Table 1), even though deep-intronic variants have been identified in more than 76 other monogenic diseases [31,32].

Deep intronic variants in *NBAS* have not been described before, and to the best of our knowledge pseudo-exon activation by altering a regulatory splicing sequence has not been reported before in patients with monogenic acute liver failure. It remains to be seen if the lack of previous reports is due to the rarity of such events or because they have been missed by current diagnostic strategies. For instance, a recent report using a targeted amplicon-based NGS panel in children with suspected monogenic hepatopathies, identified only one pathogenic heterozygous variant in 7 patients in whom autosomal recessive inheritance was suspected, thus failing to provide a definite molecular diagnosis [33]. However, this study did not include any cDNA studies and only exon-intron boundaries were targeted for sequencing. Thus, it is cases like these that are good candidates for more in-depth cDNA studies or RNA sequencing (RNA-seq) in a bid to identify a missed second causative variant that could alter splicing.

It is therefore reasonable to speculate that the diagnostic yield in monogenic causes of acute liver failure may increase using the combination of WGS and cDNA analysis to detect deep intronic variants, as illustrated in this report. Since the first description of *NBAS* as a cause of acute liver failure in 2015 [4] more than 28 patients have been described [25,28,30,34–37], and we suggest that NGS in combination with cDNA studies should be implemented early in the diagnostic work up of children with recurrent acute liver failure, especially if it is associated with fever.

Establishing a diagnosis is important to direct treatment and most importantly decide if the patient would benefit from liver transplantation. The clinical decision making related to liver transplantation in pediatric acute liver failure is not standarised [38]. It requires a multidisciplinary team to make individualized decisions, balancing the

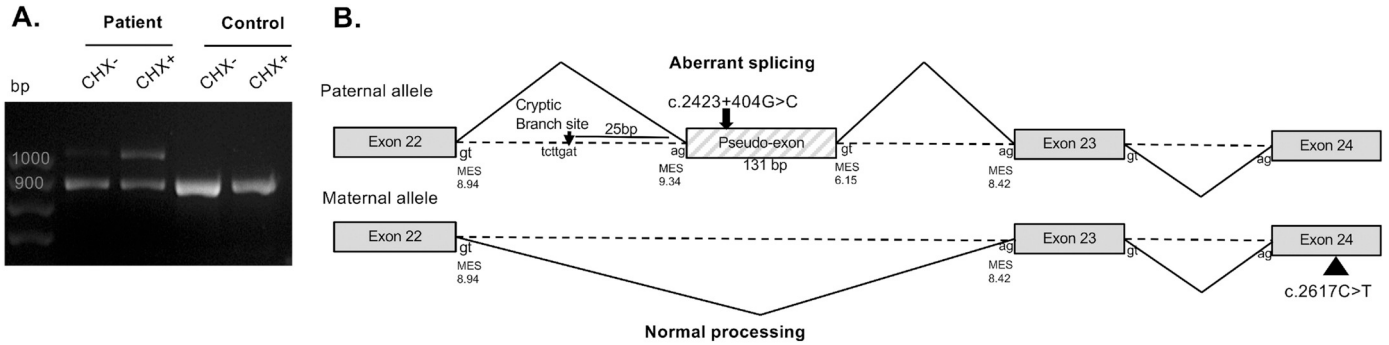

Fig. 1. A novel deep-intronic variant leads to the insertion of a pseudo-exon in *NBAS*.
A. Gel electrophoresis of RT-PCR products containing exons 18 to 25 shows a fragment of the expected size (880 bp) in both control and patient fibroblast cells as well as an additional larger fragment (~1000 bp) which is only present in the patient. This band is more prominent in the patient cells treated with cycloheximide (CHX +) to inhibit nonsense mediated decay (NMD) of the mutant transcript. B. Schematic representation of the aberrant splicing generated by the Paternally-inherited c.2423 + 404G > C deep-intronic variant, leading to inclusion of a 131 bp pseudo-exon between exons 22 and 23. The Maternally-inherited c.2617C > T;p.(Arg873Trp) variant is represented as a filled triangle and appeared homozygous in Patient cDNA generated from cells grown without cycloheximide, suggesting that the paternal transcript containing the pseudo-exon is degraded by NMD (Fig. S3). Maximum entropy scores (MES) above 3 are considered to be indicative of a splice-site (consensus values range − 20 to +20).

risks and benefits of performing a transplant, which is particularly challenging in the absence of a specific diagnosis. This is of particular concern if we take into account that acute liver failure is responsible for 11% of hepatic transplants in the USA [2], and almost half of the children with acute liver failure do not survive without this treatment [39]. In the child we described here, before the molecular diagnosis was made through WGS and cDNA studies, liver transplantation was seriously considered as a therapeutic option on several occasions. However, the patient recovered completely during episodes, making the transplant unnecessary. In addition, because of the recurrent acute liver failure, mild lactate elevation, and a low complex II + III enzyme activity in muscle there was a suspicion of mitochondrial disease as a differential diagnosis. This made the decision on liver transplantation more challenging, as patients with a multisystemic mitochondrial disease can have poor outcomes, and severe multiorgan mitochondrial disease is a contra-indication to liver transplant [2]. Fortunately,

Table 1
Selected intronic variants leading to splicing defects causing common monogenic diseases with pediatric acute liver failure.

Phenotype	Deep intronic pathogenic variants reported (ClinVar, HGMD)	Gene	Example of Variant	Consequence	References
Classic Galactosemia (MIM 230400)	No	*GALT*	c.1059 + 56C > T	54 bp insertion	[41]
Tyrosinemia type I (MIM 276700)	No	*FAH*	c.1062 + 5G > A	Skipping of exon 12	[42,43]
Hereditary fructose intolerance (MIM 229600)	No	*ALDOB*	c.-11 + 1G > C	Retention of intron 1	[44,45]
Carbamoylphosphate synthetase I deficiency (MIM 237300)	No	*CPS1*	c.3558 + 1G > C	Skipping of exon 29	[46]
Classic citrullinemia (MIM 215700)	No	*ASS1*	c.421-2A > G	Skipping of exon 7	[47]
Argininosuccinic aciduria (MIM 207900)	No	*ASL*	c.446 + 1G > A	Skipping of exon 5	[48]
Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (MIM 238970)	No	*SLC25A15*	c.56 + 1G > T	Skipping of exon 3	[49]
Wilson disease (MIM 277900)	No	*ATP7B*	c.1708-1G > C	Skipping of exon 5	[50]
Alpers-Huttenlocher syndrome (MIM 203700)	No	*POLG*	c.1251-2A > T	Skipping of exon 7	[51]
Mitochondrial DNA depletion syndrome 3 (hepatocerebral type) (MIM 251880)	No	*DGUOK*	c.444-62C > A	60 bp insertion	[52]
Mitochondrial DNA depletion syndrome 6 (hepatocerebral type) (MIM 256810)	No	*MPV17*	c.70 + 5G > A	Skipping of exon 2	[53]
Ornithine transcarbamylase deficiency (MIM 311250)	Yes	*OTC*	c.540 + 265G > A	Deep-intronic variant creates a new acceptor splice site leading to a 135 bp pseudo-exon inclusion	[54]
Niemann-Pick type C (MIM 257220)	Yes	*NPC1*	c.1554-1009G > A	Deep-intronic variant creates a new donor splice site leading to a 194 bp pseudo-exon inclusion	[55]
Infantile liver failure syndrome 2 (MIM 616483)	Yes	*NBAS*	c.2423 + 404G > C	Deep-intronic variant alters an intronic regulatory splice sequence leading to a 131 bp pseudo-exon inclusion	Present report

patients with ILFS2 tend to respond to antipyretics, high glucose and lipids during the acute episodes, and have a good overall prognosis with conservative treatment. Furthermore, when liver transplantation has been conducted during the acute episodes full recovery and no relapses have been reported [25,40].

7. Conclusion

The present study expands the spectrum of pathogenic variants causative of Infantile liver failure syndrome 2. By identifying a deep-intronic variant leading to inclusion of a pseudo-exon we describe a novel disease mechanism in the *NBAS* gene and highlight the utility of WGS and cDNA studies. We postulate that a similar approach could be conducted when only one pathogenic or likely pathogenic variant is found in a gene with a known autosomal recessive inheritance where there is a strong phenotype match. However, when no candidate variants are found, other technologies such as RNA-seq and proteomics could also aid in the diagnosis, as the current WGS bioinformatic analysis and *in silico* tools are currently inadequate for the easy identification of pathogenic deep intronic variants. This is an important area for future research as the use of NGS continues to expand and as more data are accumulated, further studies should be conducted to improve the current bioinformatic approaches to identify clinically relevant variants in non-coding regions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2018.12.002.

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