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Analysis of gene mutations in children with cholestasis of undefined etiology

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Abstract

Introduction—The discovery of genetic mutations in children with inherited syndromes of intrahepatic cholestasis allows for diagnostic specificity despite similar clinical phenotypes. Here, we aimed to determine whether mutation screening of target genes can assign a molecular diagnosis in children with idiopathic cholestasis.

Methods—DNA samples were obtained from 51 subjects with cholestasis of undefined etiology and surveyed for mutations in the genes *SERPINA1*, *JAG1*, *ATP8B1*, *ABCB11*, and *ABCB4* by a high-throughput gene chip. Then, the sequence readouts for all five genes were analyzed for mutations and correlated with clinical phenotypes. Healthy subjects served as controls.

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Results—Sequence analysis of the genes identified 14 (or 27%) subjects with missense, nonsense, deletion, and splice site variants associated with disease phenotypes based on the type of mutation and/or biallelic involvement in the *JAG1*, *ATP8B1*, *ABCB11*, or *ABCB4* genes. These patients had no syndromic features and could not be differentiated by biochemical markers or histopathology. Among the remaining subjects, 10 (or ~20%) had sequence variants in *ATP8B1* or *ABCB11* that involved only one allele, 8 had variants not likely to be associated with disease phenotypes, and 19 had no variants that changed amino acid composition.

Conclusion—Gene sequence analysis assigned a molecular diagnosis in 27% of subjects with idiopathic cholestasis based on the presence of variants likely to cause disease phenotypes.

Keywords

Liver; jaundice; PFIC; bile duct; mutation

INTRODUCTION

The evaluation of children with syndromes of intrahepatic cholestasis remains a clinical challenge despite advances in etiology and pathogenesis of diseases. Although the prevalence of individual syndromes is low, collectively they are frequent causes of chronic cholestasis. The best characterized syndromes have been linked to mutations in genes that disrupt critical cellular processes. Among these genes, disease-causing mutations have been reported in *SERPINA1* (responsible for deficiency in alpha-1-antitrypsin [A1AT]), *JAG1* (for the Alagille syndrome [AGS]), and three genes associated with progressive familial intrahepatic cholestasis (PFIC): *ATP8B1* (type 1, encoding the familial intrahepatic cholestasis-1 [FIC1] protein), *ABCB11* (type 2, encoding the bile salt export pump [BSEP]), and *ABCB4* (type 3, encoding multidrug resistance protein-3 [MDR3]) (1). Notably, the spectrum of phenotypes associated with mutations in *ATP8B1*, *ABCB11* and *ABCB4* is now broader and includes intrahepatic cholestasis of pregnancy (2-7), gallstone formation (8, 9), and hepatobiliary tumors (10-12).

Despite the lack of predominant mutational hot spots, sequence analysis of the entire coding sequence can be performed by standard capillary sequencing methods or by a hybridization-based high-throughput gene chip (13). These techniques facilitate the potential screening of mutations in the clinical setting. In one report, a mutation analysis of *ATP8B1* supported the phenotype of patients with benign recurrent intrahepatic cholestasis that were treated with a new approach to improve severe pruritus by nasobiliary drainage (14). Another mutation survey reported a previously unrecognized association between mutations in *ABCB4* and fibrosing cholestatic liver disease in adults (15). Here, we performed a comprehensive multi-gene sequence analysis to determine whether a molecular diagnosis can be assigned to children with idiopathic cholestasis. Using a chip-based resequencing methodology, we identified sequence variants in *JAG1*, *ATP8B1*, *ABCB11*, or *ABCB4* likely to cause disease phenotypes in 27% of children with cholestasis of undefined etiology based on the type and/or biallelic involvement of the sequence variants.

MATERIALS AND METHODS

Patients

We performed high-throughput nucleotide sequence analyses using peripheral blood DNA from children with idiopathic cholestasis, which was defined by the presence of persistent cholestasis (high conjugated bilirubin and high serum bile acids), normal serum levels of alpha-1-antitrypsin, absence of syndromic features, and no family history of chronic liver disease. Subjects with high serum gamma-glutamyl transpeptidase (γGTP) also had negative evaluation for main syndromic features of the Alagille syndrome (facial features, ophthalmologic examination, vertebral body anomalies, or structural cardiac defects). All subjects were either evaluated in the Pediatric Liver Care Center of Cincinnati Children's Hospital Medical Center or had blood samples, clinical data, and histopathology reports sent to our laboratory. For control subjects, DNA was also obtained from peripheral blood and used to determine the allele frequencies of new non-synonymous nucleotide variants. The controls consisted of a cohort of 50 subjects without liver disease from Southern Ohio (race: 83% White, 16% Black or African American, 1% Asian; ethnicity: 5% Hispanic or Latino). The study protocol was approved by the Institution Review Board of Cincinnati Children's Hospital Medical Center, and informed consent (and assent when appropriate) was obtained from legal guardians.

Chip hybridization and analysis

DNA was isolated from peripheral blood using the Puregene Purification Kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's protocol. Then, DNA samples served as template in long- and short-range high-fidelity PCR to amplify selected domains of target genes (amplicons), followed by hybridization with the JaundiceChip, detection of biotin-labeled signals by the GeneChip 3000 Scanner, capture with the Affymetrix GeneChip® Operating Software, and analysis with the Affymetrix GeneChip® Sequence Analysis Software (GSEQ) as described by us previously (13).

Sequence analysis

The nucleotide sequence readout for all subjects was exported into an Excel spreadsheet that also displayed the reference sequence for each gene (obtained from GenBank at www.ncbi.nlm.nih.gov/entrez) and a list of mutations associated with disease phenotypes as reported in the Human Genome Mutation Database (www.hgmd.cf.ac.uk) or in the published English literature (www.ncbi.nlm.nih.gov/sites/entrez), herein referred to as previously described nucleotide changes. All new non-synonymous variants were analyzed by the computational methods Grantham Score (16), SIFT (Sorting Intolerant From Tolerant) (17), and PolyPhen (18) to assess the likelihood of significantly modifying (or "damaging") the biological properties of encoded proteins (19). Nucleotide changes within 10 bp of the intron/exon boundaries (splice sites) were checked using NetGene (http://www.cbs.dtu.dk/biolinks/pserve2.php), a gene finder and intron splice site prediction algorithm hosted by the Center for Biological Sequence Analysis in Denmark.

Capillary sequencing

In order to validate nucleotide variants identified by the JaundiceChip, we performed capillary sequencing for every non-synonymous nucleotide substitutions, deletions, and splice site changes. Automated capillary sequencing was performed using ABI Prism® 3730 DNA Analyzer at the Gene Expression and Sequence Core at Cincinnati Children's Hospital Medical Center. Results of nucleotide sequence readouts are presented according to the nomenclature suggested by the Human Genome Variation Society (www.hgvs.org/mutnomen/).

RESULTS

Survey of mutations in subjects with cholestasis of undefined etiology

The amplification of gene fragments, probe labeling, hybridization with the chip, and analysis of the signal intensity generated nucleotide sequences for all exons and intron-exon boundaries of *SERPINA1*, *JAG1*, *ATP8B1*, *ABCB11*, and *ABCB4* in all subjects. To detect nucleotide sequence changes of potential relevance to clinical phenotypes, we analyzed the sequence output for missense variants that resulted in amino acid changes, nonsense variants, deletions/insertions, or splice site nucleotide substitutions. To be sure that sequence variants were reproducible, we re-analyzed individual variants in the patient's DNA using standard capillary sequencing. All sequence variants reported below were reproduced by capillary sequencing. From the 51 subjects with idiopathic cholestasis (or cholestasis of undefined etiology), we found two general groups of patients. One group consisted of 14 subjects that had gene sequence variants likely to cause disease phenotypes, and 10 additional subjects in whom the variations in nucleotide sequence affected only one allele of genes involved in autosomal recessive traits (thus presumably not likely to cause disease phenotypes) (Table 1). The remaining subjects either did not have sequence variations that changed amino acid composition (N=19; see Supplemental Table), had variants with high prevalence in controls, or produced amino acid changes that were predicted not to adversely impact the function of the encoded protein according to SIFT, Polyphen and Grantham scores (N=8, Table 2). Thus, from a cohort of 51 subjects with cholestasis of undefined etiology, mutation analysis enabled the assignment of a molecular diagnosis in 14 (or 27%) of subjects.

High γGTP cholestasis - Sequence variants in *JAG1* or *ABCB4*

The assignment of a molecular diagnosis in 27% of subjects was based on the presence of sequence variants in one of the genes *JAG1*, *ATP8B1*, *ABCB11*, or *ABCB4* (Figure 1). In the entire cohort, 16 subjects had high γGTP (≥100 IU/mL) and 34 had low γGTP (<100 IU/mL); γGTP was not available in one subject. Among those with high γGTP, two subjects displayed *JAG1* variants. They had no evidence of typical facial features or ocular, cardiovascular, or vertebral body abnormalities. Liver biopsy was done in one of them at 3.5 months of age and showed canalicular cholestasis, giant cell transformation and small bile ducts (Table 3). In these patients, the *JAG1* variants introduced a premature stop codon (p.C251X) or resulted in an amino acid substitution that is predicted to be damaging to the function of the encoded protein (p.V1086E; Figure 2). Both variants involved one allele, which were consistent with the autosomal dominant mode of inheritance for subjects with

the Alagille syndrome. One other patient without syndromic features and with high γGTP had liver biopsy at 1.5 years of age, which showed pseudoacinar transformation of hepatocytes, portal inflammation, and moderate fibrosis. This patient had one sequence variant that introduced a premature stop codon (p.Q945X) in *ABCB4* and a second missense variant predicted to be damaging to the encoded protein (p.Y1171C; Table 3 and Figure 2), which together are likely to result in MDR3 deficiency.

Low γGTP cholestasis - Sequence variants in *ATP8B1* or *ABCB11*

The remaining 11 subjects had γGTP below 100 IU/μL. In this group, 3 had *ATP8B1* biallelic variants that included homozygous deletions, missense, and splice site changes consistent with deficiency of the encoded FIC1 protein (Table 3). One patient with compound heterozygous variants (c.1819+1g>a/p.R930X) subsequently had electron microscopy of a liver biopsy with features consistent with Byler's bile in the canaliculi, while the other two subjects had canalicular and cytoplasmic cholestasis, portal inflammation and fibrosis (Table 3). The remaining 8 subjects, which comprised the majority of the subjects in this group (Figure 1), had *ABCB11* sequence variants that included homozygous or compound heterozygous deletions, missense and nonsense changes; these variants are in keeping with deficiency of the encoded BSEP protein. In these subjects, liver histopathology reports did not allow differentiation from the patients with *ATP8B1* variants (Table 3). Thus, mutation survey in the low γGTP group enabled the potential assignment of subjects into specific diagnosis despite similar clinical, biochemical, and histological features.

Heterozygous variants and intrahepatic cholestasis

Mutation survey also identified heterozygous sequence variants in *ATP8B1* and *ABCB11* in 10 of 51 (or ~20%) subjects with intrahepatic cholestasis (Figure 1). The involvement of only one allele does not support a direct link with disease phenotype due to the autosomal recessive pattern of inheritance of mutations in both genes. Two of the sequence variants in *ATP8B1* and three in *ABCB11* were reported previously in subjects with intrahepatic cholestasis of pregnancy or benign recurrent intrahepatic cholestasis [for *ATP8B1*; references (20, 21)] or PFIC2 [for *ABCB11*; references (10, 22, 23)], while the new variants had low allele incidence in the control population (Table 4). Of note, the presence of high levels of γGTP in one subject with an *ATP8B1* variant and in two with *ABCB11* variants is consistent with a lack of causality with liver disease secondary to deficiencies of these canalicular transporters.

DISCUSSION

We found sequence variants in one of the *JAG1*, *ATP8B1*, *ABCB11*, or *ABCB4* genes in 14 of 51 (or 27%) subjects with chronic cholestasis of undefined etiology. The variants were homozygous or compound heterozygous for *ATP8B1*, *ABCB11*, and *ABCB4* or heterozygous for *JAG1*; all were reported previously in subjects with well defined clinical phenotypes, or represent new variants predicted to be damaging to the encoded mutant protein. Although another 20% of the cohort carried heterozygous variants in *ATP8B1* or *ABCB11* likely to impair the function of the encoded protein, the involvement of only one allele does not

support a causative association based on the autosomal recessive mode of inheritance for mutations in these genes. Most interesting, for the entire cohort, 50 of 51 subjects with idiopathic chronic cholestasis had no clinical, biochemical, or histological features that enabled the clinical diagnosis of either Alagille syndrome or one of the syndromes caused by deficiency of FIC1, BSEP, or MDR3. Thus, these data suggest that mutation surveys of candidate genes may enable a molecular diagnosis based on the presence of nucleotide sequence variants likely to be associated with disease phenotypes, even when a predominant clinical, biochemical, or histological pattern is not obvious.

A careful analysis of clinical features, biochemical markers (such as the levels of serum γGTP in children with other markers of cholestasis) and histopathology often narrows the diagnosis to a small number of syndromes of intrahepatic cholestasis [reviewed in (1, 24)]. If typical facial features associated with ocular, cardiovascular, and vertebral abnormalities are present, the diagnosis of Alagille syndrome is in order. However, the diagnosis may not be obvious in young infants with few or incomplete syndromic features or with a biopsy without paucity of bile ducts. In our cohort, two infants with these features (ages: 1 and 3.5 months) were found to carry *JAG1* variants. The finding of these two patients probably represents an underestimation because the chip-based gene sequencing may not detect heterozygous deletions or insertions (13), which may account for a substantial percent of mutations reported in subjects with the Alagille syndrome (25). In contrast, the finding of ABCB4 variants in only 1 of 16 subjects with high γGTP levels is consistent with a low incidence of MDR3 deficiency in the cohort because nucleotide changes are detected by the chip and are the most frequent types of mutations in these patients.

Among the subjects with low γGTP levels, the most common biallelic variants affected *ABCB11*, which encodes for BSEP. These patients were indistinguishable from those with *ATP8B1* variants. The spectrum of sequence variants included missense and nonsense mutations, splice site changes, and homozygous deletions, all reported previously in patients with the diagnosis of PFIC1 or 2, respectively, or were new and predicted to impact the function of the mutant protein. The finding that 19 of 51 subjects displayed no candidate mutation in *JAG1, ATP8B1, ABCB11,* or *ABCB4* suggests that a molecular diagnosis cannot be ascertained in a substantial portion of children with undefined cholestasis. These patients may constitute a population that is most suitable for gene sequencing studies to identify new cholestasis-related genes. Before such endeavor, it would be important to complement chip-based sequencing with complementary sequencing technologies (examples: genome and cDNA capillary sequencing, fluorescence in situ hybridization, real-time PCR) in the same subjects to precisely show the absence of other nucleotide sequence changes in these genes.

The cholestasis phenotype in the subjects with heterozygous mutations of *ATP8B1* and *ABCB11* cannot be explained solely by the nucleotide changes reported here. It is possible that a second mutation may reside in promoter regions or intron domains not sequenced by the chip, or that insertions or deletions in the other allele were not detected by the chip. Another possibility is the co-existence of a heterozygous mutation in one of the other four related gene sequenced by the chip. Our experimental strategy formally rejected this scenario. However, it remains possible that mutations in other genes not included in the chip may contribute to the clinical phenotype.

Although mutation survey may represent a powerful ancillary test to improve specificity of diagnostic algorithms, it is important to recognize that no one single technology reported to date is 100% accurate in identifying all possible mutations. In one study, the combination of four different techniques was necessary to increase accuracy to 94% for mutations in *JAG1* in subjects with carefully defined features of Alagille syndrome (25). Without a highly prevalent mutation in most patients with inherited syndromes of intrahepatic cholestasis involving *JAG1*, *ATP8B1*, *ABCB11*, and *ABCB4*, the findings of new mutations spread across the entire genes would benefit from functional analysis of the mutant protein or complementary immunohistochemical analysis to more precisely assess the impact of candidate mutations to the function of the protein. Despite these limitations, our data suggest that an analysis of the nucleotide composition of candidate genes identifies gene sequence variants associated with disease phenotypes. Whether this approach is used in clinical practice or as an investigational tool, it has the potential to broaden our knowledge of the genetic basis of cholestatic syndromes and the design of patient-based studies that take into account the genetic makeup of the individual patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A1AT	Alpha-1-antitrypsin
AGS	Alagille syndrome
PFIC	Progressive familial intrahepatic cholestasis
FIC1	Familial intrahepatic cholestasis-1
BSEP	Bile salt export pump
MDR3	Multidrug resistance protein-3
GSEQ	GeneChip® sequence analysis software
SIFT	Sorting intolerant from tolerant

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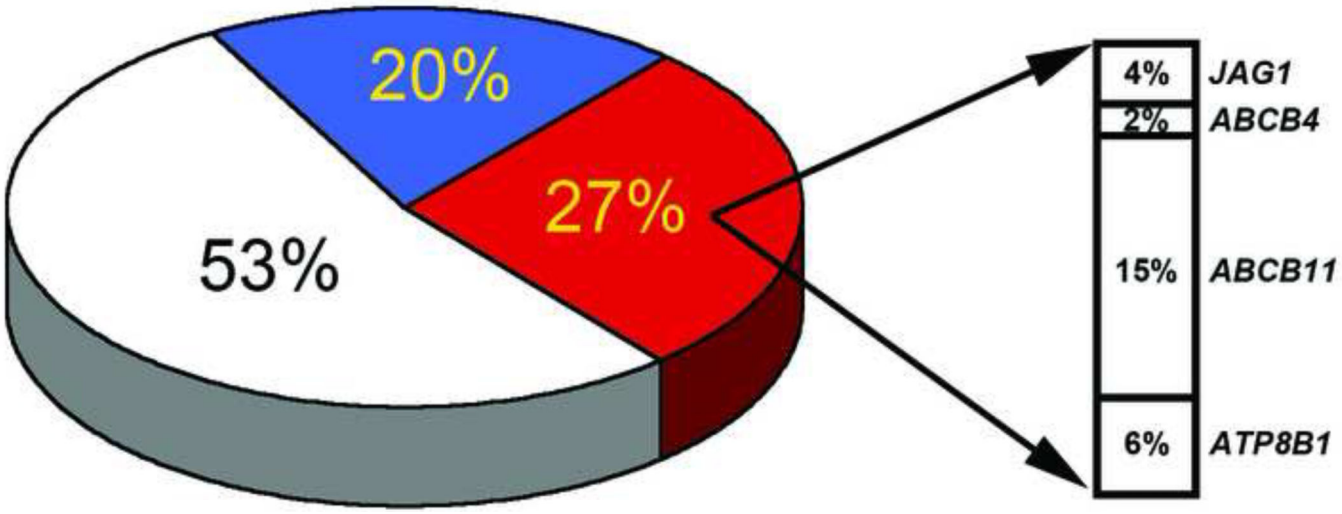

Figure 1.

Pie diagram depicting the percentage of subjects displaying sequence variants in a cohort of 51 subjects with cholestasis of undefined etiology. All variants in the red portion were homozygous or compound heterozygous, except for those involving *JAG1* (see percentage in vertical bar), while those in the blue portion were heterozygous for ATP8B1 or ABCB11. Percent in the white portion corresponds to subjects without sequence variants or with variants of high prevalence in controls.

Gene	Mutation	Grantham	SIFT	Polyphen
JAG1	p.V1086E	121	0.01	1.926
ABCB4	p.Y1171C	194	0	3.15
ATP8B1	p.I1050K	102	0.01	1.826
	p.R930Q	43	0.23	1.14
ABCB11	p.T87R	71	0	2.217
	p.M123T	81	0.56	1.4
	p.E1223D	45	0.01	0.99
	p.C68Y	194	0.19	2.517
	p.R832H	29	0	2.934
	p.G556R	125	0	2.667
	p.D1243G	94	0	3.051

Figure 2.

Categorization of new nucleotide variants identified in subjects with cholestasis of undefined etiology according to the Grantham Score, SIFT, and PolyPhen (damaging predicted by scores of >100, <0.05 and >1.5, respectively). The green color predicts the variant to be “benign” and the red color as “damaging” to the function of the encoded protein when the nucleotide substitutions affect both alleles for *ATP8B1*, *ABCB11*, and *ABCB4* or one allele for *JAG1*.

Table 1

Type and frequency of gene sequence variants in a cohort of 51 subjects with cholestasis of undefined etiology.

Type of mutation	Association with disease	Number (% of 51)
Missense, nonsense, deletion, splice site (2 alleles)	Predicted	14 (27%)
Missense (1 allele, rare in controls))	Uncertain	10 (20%)
Missense (1 allele, prevalent in controls*)	No	8 (16%)
None	Not applicable	19 (37%)

* Not likely to cause disease phenotype because of high allele frequency in normal controls or encode the PiI or PiS alleles of A1AT, which do not independently cause an abnormal hepatic phenotype

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Table 2

Description of subjects with cholestasis of undefined etiology (CUE*) found to have heterozygous variants with high incidence in normal controls or encoding amino acid changes not predicted to alter the function of the encoded protein**.

Subject*	Age	γGTP	Liver Biopsy	Gene	Mutation	Comment
CUE-1	10 m	77	GCT, proliferation of bile ducts	*JAG1*	p.R900Q	New, not damaging*
CUE-2	5 m	150	Canalicular cholestasis, GCT, bile duct proliferation, portal fibrosis	*ATP8B1*	p.I393V	15% of controls
CUE-3	13.5 yr	46	Cholestasis, bile duct paucity, mild portal fibrosis	*ABCB4*	pT175A	3.2% of controls (9, 26)
CUE-4	4 yr	N.A.	Cholestasis, GCT	*SERPINA1*	p.R39C	PiI allele
CUE-5	6 yr	517	Portal and central fibrosis	*SERPINA1*	p.E264V	PiS allele
CUE-6	5 m	80	Not done	*SERPINA1*	p.E264V	PiS allele
CUE-7	3 m	66	GCT, portal inflammation and fibrosis, bile duct proliferation	*SERPINA1*	p.R39C	PiI allele
CUE-8	2 m	86	Canalicular cholestasis, no fibrosis	*SERPINA1*	p.R39C	PiI allele

*CUE: All patients underwent systematic clinical, biochemical, and histological analysis. Those patients who had γGTP >100 also had negative investigation for main syndromic features of the Alagille disease (facial features, ophthalmologic examination, vertebral body anomalies, or structural cardiac defects).

Table 3

Gene sequence variants likely to cause disease phenotypes in subjects with cholestasis of undefined etiology (CUE*).

Subject	Age	γGTP	Liver Biopsy	Gene	Variant (allele frequency, if new)	Reference
CUE-1	1 m	458	Not done	*JAG1*	p.C251X	New
CUE-2	3.5 m	632	Cholestasis, GCT, small bile ducts	*JAG1*	p.V1086E	New
CUE-3	1.5 yr	400	Pseudoacinar transformation, portal inflammation, moderate fibrosis	*ABCB4*	p.Q945X/p.Y1171C (0%)	New/New
CUE-4	26 yr	47	Cytoplasmic and canalicular cholestasis, portal fibrosis	*ATP8B1*	p.N45T/p.I1050K (0%)	(20)/New
CUE-5	3.5 yr	40	Electron microscopy consistent with Byler's disease	*ATP8B1*	c.1819+1g>a/p.R930X	New**/(27)
CUE-6	1.5 yr	20	Canalicular cholestasis, portal inflammation	*ATP8B1*	g.92918del565/g.92918del565	(13)
CUE-7	1 yr	44	GCT, portal inflammation and fibrosis	*ABCB11*	p.R928X/p.R1090X	(13), (28)
CUE-8	2.5 yr	62	Canalicular cholestasis, periportal inflammation, portal fibrosis	*ABCB11*	p.I541T/p.I541T	(12)
CUE-9	5.5 yr	54	Cholestasis, GCT, ductopenia, briding fibrosis	*ABCB11*	p.E297G/E297G	(22)
CUE-10	11 yr	9	Minimal cholestasis; insufficient representation of portal tracts	*ABCB11*	p.R948C/p.E1223D (0%)	(36)/New
CUE-11	6 m	64	Cytoplasmic and canalicular cholestasis, GCT, fibrosis	*ABCB11*	p.C68Y (0%)/p.R832H (0%)	New, New
CUE-12	2 yr	42	Not done	*ABCB11*	c.3770delA/c.3770delA	New**/New**
CUE-13	19 yr	29	Marked cholestasis, portal fibrosis.	*ABCB11*	c.3929delG/c.3929delG	New**/New**
CUE-14	3 yr	44	Canalicular cholestasis, portal-portal bridging fibrosis	*ABCB11*	p.G556R (0%)/p.D1243G (0%)	New, New

γGTP: gamma-glutamyl transpeptidase; GCT: giant cell transformation

*CUE: All patients underwent systematic clinical and biochemical analysis, and most had a liver biopsy (shown above). Those patients who had γGTP >100 also had negative investigation for main syndromic features of the Alagille disease (facial features, ophthalmologic examination, vertebral body anomalies, or structural cardiac defects).

**Variants that introduce a stop codon or deletions were not tested in normal subjects because they are likely to alter function of encoded protein.

Table 4

Gene sequence variants involving one allele in subjects with cholestasis of undefined etiology (CUE*). The allele prevalence for new sequence variants were determined in controls.

Subject	Age	γGTP	Liver Biopsy	Gene	Variant (allele frequency, if new)	Reference
CUE-1	1.5 yr	17	Bile duct paucity, cholestasis, GCT	*ATP8B1*	p.N45T	(20)
CUE-2[a]	13.5 yr	11	No biopsy data	*ATP8B1*	p.I661T	(21)
CUE-3	2 yr	399	No biopsy data	*ATP8B1*	p.N45T	(20)
CUE-4	2 wk	85	Cytoplasmic cholestasis, GCT	*ATP8B1*	c.3016-9c>a (2%)	New
CUE-5	1 m	60	Not done	*ATP8B1*	p.R930Q (0.2%)**	New
CUE-6[a]	14 yr	12	Canalicular cholestasis, GCT, portal inflammation and fibrosis	*ABCB11*	p.R1153C	(22)
CUE-7	2.5 yr	21	Marked lobular cholestasis, GCT, portal inflammation and fibrosis	*ABCB11*	c.2138-8t>g	(10)
CUE-8	7 yr	338	No biopsy data	*ABCB11*	p.R487H	(23)
CUE-9	5.5 yr	173	Cholestasis, periportal inflammation, portal fibrosis	*ABCB11*	p.T87R (0%)	New
CUE-10	2.5 yr	65	Pseudoacinar transformation, portal inflammation and fibrosis	*ABCB11*	p.M123T (0%)	New

γGTP: gamma-glutamyl transpeptidase; GCT: giant cell transformation

*CUE: All patients underwent systematic clinical and biochemical analysis, and most had a liver biopsy (shown above). Those patients who had γGTP >100 also had negative investigation for main syndromic features of the Alagille disease (facial features, ophthalmologic examination, vertebral body anomalies, or structural cardiac defects).

**Not predicted to be damaging to the function of the encoded protein based on SIFT, Polyphen, and Grantham scores (Figure 2).