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ORIGINAL ARTICLE

Diagnostic yield and novel candidate genes by next generation sequencing in 166 children with intrahepatic cholestasis

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Abstract

Background and aims Cholestatic liver disease is a leading referral to pediatric liver transplant centers. Inherited disorders are the second most frequent cause of cholestasis in the first month of life.

Methods We retrospectively characterized the genotype and phenotype of 166 participants with intrahepatic cholestasis, and re-analyzed phenotype and whole-exome sequencing (WES) data from patients with previously undetermined genetic etiology for newly published genes and novel candidates. Functional validations of selected variants were conducted in cultured cells.

Results Overall, we identified disease-causing variants in 31% (52/166) of our study participants. Of the 52 individuals, 18 (35%) had metabolic liver diseases, 9 (17%) had syndromic cholestasis, 9 (17%) had progressive familial intrahepatic cholestasis, 3 (6%) had bile acid synthesis defects, 3(6%) had infantile liver failure and 10 (19%) had a phenocopy of intrahepatic cholestasis. By reverse phenotyping, we identified a de novo variant c.1883G > A in *FAM111B* of a case with high glutamyl transpeptidase (GGT) cholestasis. By re-analyzing WES data, two patients were newly solved, who had compound heterozygous variants in recently published genes *KIF12* and *USP53*, respectively. Our additional search for novel candidates in unsolved WES families revealed four potential novel candidate genes (*NCOA6*, *CCDC88B*, *USP24* and *ATP11C*), among which the patients with variants in *NCOA6* and *ATP11C* recapitulate the cholestasis phenotype in mice models.

Conclusions In a single-center pediatric cohort, we identified monogenic variants in 22 known human intrahepatic cholestasis or phenocopy genes, explaining up to 31% of the intrahepatic cholestasis patients. Our findings suggest that re-evaluating existing WES data from well-phenotyped patients on a regular basis can increase the diagnostic yield for cholestatic liver disease in children.

Keywords Intrahepatic cholestasis · Exome sequencing · Hepatocytes · Cholangiocytes · Reverse phenotyping · *NCOA6* · *ATP11C*

Introduction

Cholestatic liver disease refers to a group of uncommon but potentially serious disorders that affect bile formation or flow, causing elevated levels of serum conjugated bilirubin, glutamyl transpeptidase, phosphatase or serum bile acids [1]. It is a leading cause of referral to pediatric liver transplant centers, affecting approximately 1 in 2500 term infants worldwide [1, 2]. According to data from the European liver transplant registry, cholestatic liver disease is the primary indication for liver transplant in 70.6% of children aged 0–2 years and 33.8% of those aged 2–18 years.

The growing recognized monogenic disorders account for predominant 25% of neonatal cholestasis [1]. The clinical presentation of monogenic cholestasis broadly overlaps

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making etiology diagnosis challenging. Next generation sequencing (NGS) provides a timely and comprehensive assessment of the genotype, facilitating accurate diagnosis and improved patient outcomes. The latest guideline recommended the use of NGS in the proper clinical context [1]. The reported yield of NGS for pediatric intrahepatic cholestasis is variable (12%–33.7%), in part due to the differences of targeted known gene lists [3–5]. Whole exome sequencing (WES) overcomes this bottleneck, enabling reanalysis of newly reported genes in unsolved patients, and particularly, turning out an additional advantage to identify causal variants in novel candidate genes [6].

In this study, we conducted a systematic analysis of genetic and phenotypic data from 166 children with cholestatic liver disease. We focused on evaluating recently published genes that were not recognized as cholestasis-related at the time of clinical testing in unsolved families. We demonstrated the utility of ongoing research analysis of WES data, which led to the identification of novel genetic etiology for a substantial unsolved proportion of our cohort. Furthermore, we fully evaluated potential novel candidate genes in genetically undiagnosed families, considering their expression in hepatocytes and cholangiocytes, available resembling phenotype from existing mice models, and involvement of known biological, physiological, and functional relevance to hepatobiliary dysfunction derived cholestatic diseases.

Materials and methods

Patients

We retrospectively reviewed the clinical features and molecular findings of 166 unrelated patients with intrahepatic cholestasis at Children's Hospital of Nanjing Medical University from 2017 to 2021. The inclusion criteria in this study were: (i) a direct bilirubin value greater than 1.0 mg/dL or high glutamyl transpeptidase, phosphatase, serum bile acids that indicate cholestasis [1]; (ii) the specific request from clinicians to perform diagnosis at molecular level; (iii) the age of the patient is $\leq$ 18 years; (iv) signed informed consent to perform molecular investigations. The exclusion criteria in this study were: congenital biliary atresia, tumor, primary biliary cholangitis, primary sclerosing cholangitis, confirmed causative infections including hepatitis viral (cytomegalovirus, herpes virus, Epstein-Barr virus, hepatitis virus), treponema pallidum, and sepsis and urinary tract infection.

The signs considering to genetic testing in this cohort are as follows: (i) the patient's mother with cholestasis of pregnancy, acute fatty liver or cholelithiasis of pregnancy; (ii) alcoholic stool excluded biliary atresia; (iii) family history of hereditary metabolic liver disease; (iv) complicated with cardiovascular dysplasia, skeletal dysplasia, or ocular abnormalities; (v) dysmorphic facies; (vi) splenomegaly; (vii) neurological abnormalities (irritability, lethargy, poor feeding, hypotonia or seizures); (viii) abnormal in newborn inherited metabolic diseases screening; (ix) poor response to 1–2 weeks of ursodeoxycholic acid (UDCA) treatment. The number of patients enrolled for each of the above nine signs to genetic testing is shown in Supplementary Figure S1.

Clinical and laboratory data

The clinical data included age at onset, age at diagnosis, sex, symptoms, family history, positive signs. The biochemical data included alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum total bilirubin (TBIL), serum conjugated bilirubin (DBIL), glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), serum albumin (ALB), bile acid, prothrombin time (PT), hemoglobin, platelet count and hepatitis viral serologies (cytomegalovirus, herpes virus, Epstein-Barr virus, negative hepatitis B surface antigen and antibody to hepatitis B core antigen, hepatitis C virus). Part of the patients underwent ferritin, iron studies, ceruloplasmin, antinuclear antibody, alpha-1-antitrypsin, abdominal ultrasound, cardiac ultrasound, spinal X-ray, and liver biopsy.

Treatment and follow-up

UDCA at the dose of 10 ~ 20 mg/kgd and liposoluble vitamin were given to all the patients. According to the different causes, specific treatments were given to different patients, such as lactose-free formula to the patients with citrin deficiency, coagulation factor to the patients with severe coagulation disorders, non-biological artificial liver support to the patients with liver failure (the detail shows in Supplementary Table S1). The biochemical data and liver ultrasound at each visit from the time of diagnosis to the time of inclusion in the study were also recorded.

Next generation sequencing (NGS) and variant calling

93 patients were screened by an NGS based panel including 210 genes that are currently known to cause intrahepatic cholestasis (Supplementary Table S2). All targeted regions including exons and exon–intron boundaries (include 50 base pairs at each end) of the 210 genes were captured using a GenCap kit (MyGenostics GenCap Enrichment technologies). The enrichment libraries were sequenced on Illumina HisSeq 2500 sequencer for paired read 150 bp.

73 patients underwent whole exon sequencing evaluation. WES was performed as previously described [7]. In brief, genomic DNA was sheared into fragments and then

hybridized with the xGen Exome Research Panel v1.0 probe sequence capture array from IDT (Integrated Device Technology, USA) to enrich the exonic region. The enriched libraries were analyzed on an Illumina HiSeq XTen (Illumina, USA) platform. Low-quality variations of the quality score < 20 (Q20) were filtered out. Sequencing reads were mapped to the GRCh37/Hg19 reference genome via Burrows-Wheeler Aligner (BWA) software.

Variant filtering and evaluation

All identified variants were annotated using the 1000 Genomes Project (Chinese), dbSNP, and Genome Aggregation Database (gnomAD). Variants with a minor allele frequency higher than 1% were filtered out. Synonymous exonic and all intronic variants that were not located within splice site regions were excluded. Retained variants, which included nonsynonymous variants and all strong splice site variants, were then further evaluated using the ACMG (American College of Medical Genetics and Genomics) criteria [8] and further validated by direct Sanger sequencing.

Identification of novel genetic causes by familial analysis

WES full evaluation included filtering of the single nucleotide variants and indels with segregated de novo, homozygous/compound heterozygous, or maternally inherited X-linked variants. Candidate intrahepatic cholestasis genes selected based on biological, physiological, expressive and functional relevance to hepatobiliary dysfunction derived cholestatic diseases, but for which no known human disease association yet exists.

Functional validations of selected variants

Wild type and mutant plasmids construction, HEK293 cell culture, immunoblotting, immunofluorescence, and luciferase assays were performed to evaluate functional consequences of *NCOA6* variants (Supplementary Methods).

Statistical analysis

Statistical analyses were performed by SPSS version 23.0 software (IBM, Corp., Armonk, NY, United States). Descriptive count data are represented by median (minimum–maximum). Between-group comparisons were performed using Fisher's exact test or Mann–Whitney *U* test. Two-tailed $p < 0.05$ was considered to indicated statistical significance.

Web resources

1000 Genomes Browser http://browser.1000genomes.org

Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustal

European liver transplant registry, http://www.eltr.org/-Results-.html

Genome Aggregation Database (gnomAD), http://gnomad.broadinstitute.org

HGMD Professional 2016.3, https://portal.biobase-international.com/hgmd

MutationTaster http://www.mutationtaster.org

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

Polyphen2, http://genetics.bwh.harvard.edu/pph2

Sorting Intolerant from Tolerant (SIFT), http://sift.jcvi.org

Results

Diagnostic workflow

A total of 166 patients with intrahepatic cholestasis of unknown etiology were enrolled in this cohort between 2017 and 2021(Fig. 1). Of the 166 probands, 93 patients underwent panel capture-based targeted sequencing, and 73 patients were screened using WES, including 29 families who underwent trio-WES. At the time of clinical testing, 49 probands received a definite molecular diagnosis. The unsolved WES patients were followed up, re-analyzed for phenotype and WES data, and three of them were newly diagnosed. Moreover, 46 unsolved WES families were evaluated, and four novel candidate genes were proposed.

Cohort characteristics

The male-to-female ratio of the 166 patients was 52:114. The median age at onset was one month (range, 2 days to 13.5 years old). The majority of the 166 patients were younger than one year old (131/166). The clinical characteristics of the 166 intrahepatic cholestasis children are summarized in Table 1.

Diagnostic yield

Overall, we identified pathogenic or likely pathogenic(P/LP) causative variants in 26% of the patients, and variants of uncertain significance (VUS) in 14% of the patients (Supplementary Table S1). The diagnostic yield in this cohort was 31.3% ($n = 52/166$), representing 22 genes (Fig. 2). The diagnostic yield by trio, WES, and panel were 37.9%, 36.4%, 26.9%, respectively (Fig. 2). The top six mutated genes of definite diagnosed patients in this cohort, in order, were:

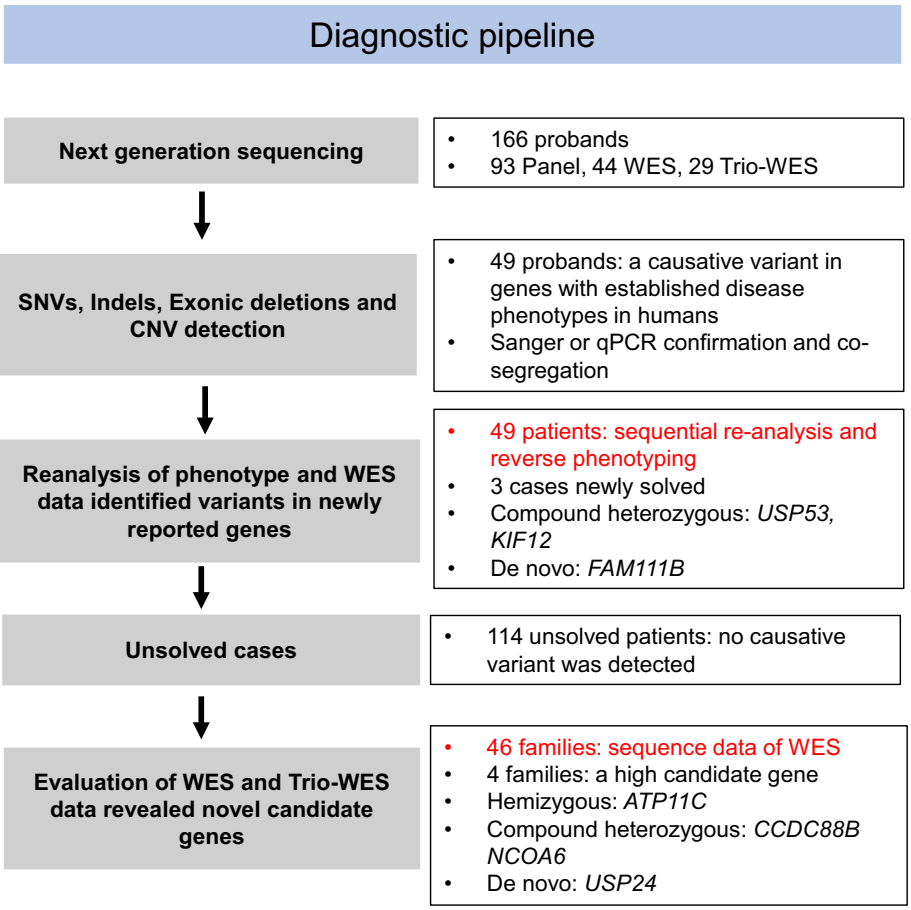

Fig. 1 Overall workflow of our diagnostic pipeline. CNV, copy-number variation; Indel, insertion/deletion; SNV, single nucleotide variant; Panel, intrahepatic cholestasis gene panel sequencing; WES, whole-exome sequencing; Trio-WES, trio based WES

Table 1 Characteristics of 166 pediatric patients with intrahepatic cholestasis

Characteristics	Total ($N=166$)	Solved ($N=52$)	Unsolved ($N=114$)	p value
Sex (male/female)	114/52	32/20	82/32	0.208
Familial/sporadic cases	5/161	3/49	2/112	1.000
Age at onset (range)	1 month (2 days, 13.5 years)	1 month (2 days, 13 years)	1 month (2 days, 13.5 years)	0.876
Hepatomegaly and/or splenomegaly (n/N)	34/166	19/52	15/114	0.096
Extrahepatic involvement (n/N)	64/166	28/52	36/114	0.010*
Alanine aminotransferase (U/L)	101 (3,3846)	60 (9,3846)	119.5 (3,3050)	< 0.001**
Aspartate aminotransferase (U/L)	145.5 (22,11,189)	116 (22,11,189)	168 (22,3625)	0.002*
Glutamyl transpeptidase (U/L)	112.5 (8,1155)	176 (8,1155)	95.5 (15,621)	0.014*
Total bilirubin (μmol/L)	118.9 (15.5,536.8)	129.4 (30.2,390.2)	114.4 (15.5,536.8)	0.384
Direct bilirubin (μmol/L)	90 (5.3,431.2)	90.9 (18.2,325.9)	90 (5.3,431.2)	0.924
Albumin (g/L)	39.7 (19,50.2)	39.8 (21.4,47.4)	39.8 (19,50.2)	0.129
Hemoglobin (g/L)	105.9 ± 17.1	102.2 ± 14.8	107.6 ± 17.9	0.060

*$p < 0.05$; **$p < 0.001$

SLC25A13, *JAG1*, *ABCC2*, *SLC10A1*, *ABCB11*, *ABCB4* (Fig. 2). As shown in Fig. 2, the 52 patients with an established monogenic diagnosis caused by variants in 22 genes can be divided into six categories: metabolic liver disease (*SLC25A13*, *NPC1, ATP7B,* 18/52), syndromic cholestasis (*JAG1, FAM111B*, 9/52), progressive familial intrahepatic cholestasis (*ATP8B1, ABCB11, ABCB4, TJP2*, *USP53, KIF12, VPS33B,* 9/52), inborn errors of bile acid synthetic defects (*HSD3B7, AKR1D1, CYP27A1*, 3/52), infantile liver failure (*LARS, NBAS, TRMU*, 3/52), and phenocopy of intrahepatic cholestasis(*ABCC2, SLCO1B1/SLCO1B3, SLC10A1, ANK1,* 10/52). The detailed phenotypic and follow-up results

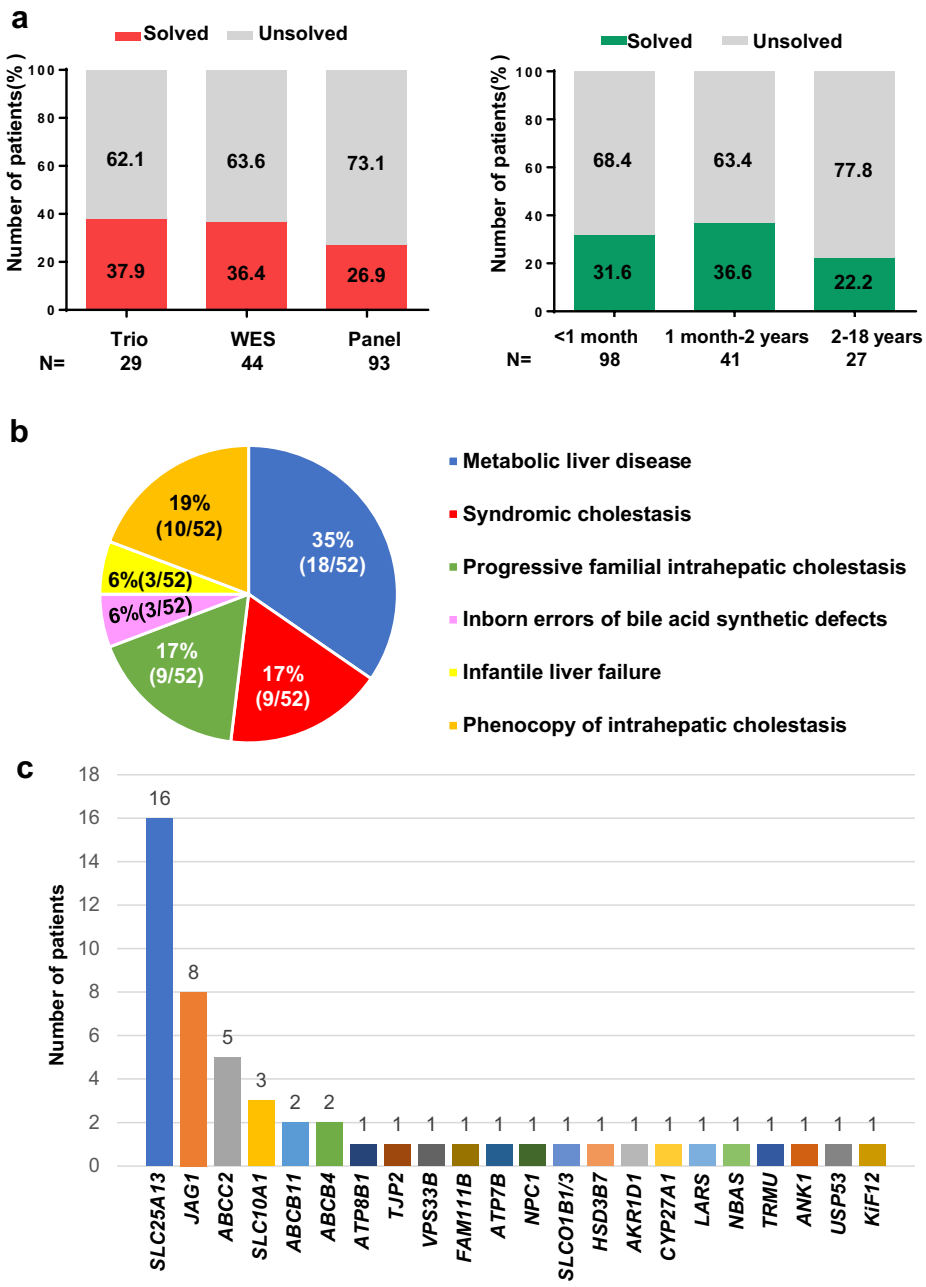

Fig. 2 Diagnostic yield and mutated monogenic genes of 166 patients with intrahepatic cholestasis in which a causative gene was detected by exome sequencing. **a** Numbers and percentages of 166 children with intrahepatic cholestasis in which a causative variant in a known monogenic cholestasis gene was detected by different sequencing strategies (left panel) and in different age groups (right panel). Trio, trio-based whole exome sequencing; WES, whole exome sequencing; Panel, panel sequencing. **b** Pie chart showed the grouping and distribution of inherited intrahepatic cholestasis into six categories in our cohort. The monogenic disorders caused by variants in 22 genes can be divided into six categories: metabolic liver disease (blue), syndromic cholestasis(red), progressive familial intrahepatic cholestasis(green), inborn errors of bile acid synthetic defects(pink), infantile liver failure(yellow) and phenocopy of intrahepatic cholestasis(orange). **c** The numbers of patients with inherited intrahepatic cholestasis for 22 monogenic causes identified in our cohort

of the 49 patients who received a molecular diagnosis at the time of clinical testing were shown in the Supplementary data.

Phenotype features

Compared with the unsolved group, the patients in the solved group had a higher extrahepatic manifestations rate and higher AST/GGT levels but lower ALT levels. However, there was no significant difference between the two groups in gender, age distribution of onset, hepatosplenomegaly, and other laboratory data, including TBIL, DBIL, ALB, and HB (Table 1).

Retrospective analysis for molecular diagnosis

We conducted a retrospective analysis of WES data to identify newly published morbid genes and facilitate the molecular diagnosis in 3 out of 166 patients. Our analysis involved reverse phenotyping, and the details were described below.

In the first case, we identified compound heterozygous variants (c.91G > A;p.Gly31Ser /c.394C > T;p.His132Tyr) in *USP53* in a patient who presented with normal GGT

cholestasis, prolonged prothrombin time, ecchymosis, and anemia (Supplementary Figure S2). At the age of 7 months, the patient's symptoms improved after treatment with coagulation factor, liposoluble vitamin, and UDCA, and no extrahepatic manifestation was noted.

In the second case, we diagnosed a patient with infant onset high GGT cholestasis with *KIF12* deficiency, with compound heterozygous variants (c.538C > T;p.Arg180Trp /c.539G > A; p.Arg180Gln in *KIF12*) (Fig. 3). The patient developed splenomegaly at the age of 4, and experienced gastroesophageal variceal hemorrhage at the age of 6, leading to endoscopic sclerotherapy. A liver biopsy showed nodular cirrhotic with scarce lymphomononuclear cell infiltration and paucity of intrahepatic bile duct. The patient underwent liver transplantation at the age of 6.5 due to recurrent gastroesophageal variceal hemorrhage.

In the third case, we diagnosed a 2-year-old girl with neonate onset high GGT cholestasis who gradually improved with age but suffered from progressive poikiloderma and alopecia. With reverse phenotyping, we identified a de novo pathogenic variant (c.1883G > A(p.Ser628Asn)) in the *FAM111B* gene (Supplementary Figure S3). The residue is well conserved and located at the loop link region between the two Trypsin-like cysteine/serine peptidase domains.

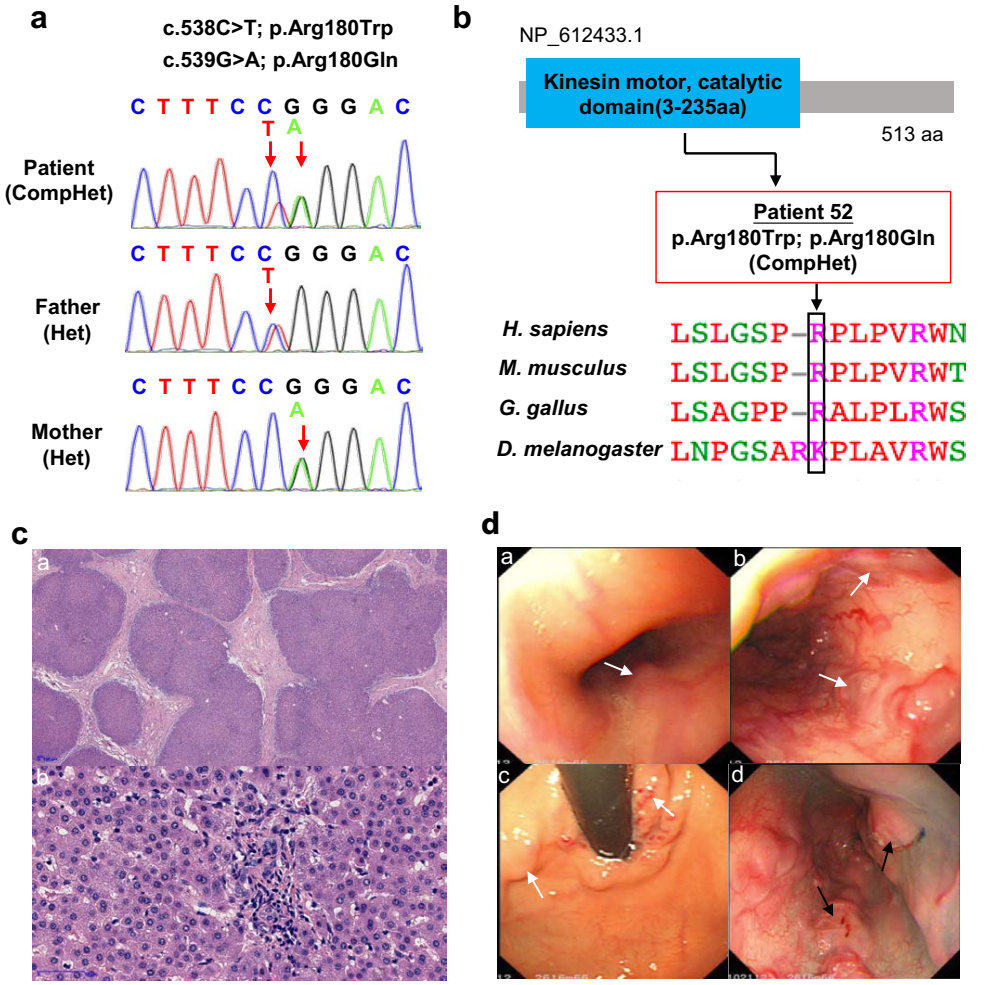

Fig. 3 Whole exome sequencing identified compound heterozygous variants of *KIF12* in a patient with high GGT cholestasis. **a** Sequencing chromatograms of the compound heterozygous variants: c.538C > T; p.Arg180Trp and c.539G > A; p.Arg180Gln of the *KIF12* gene in the patient 52 presenting with high GGT intrahepatic cholestasis. **b** Protein domain content of KIF12 and multiple sequence alignment of the KIF12 protein region flanking residue Arg108. The Arg108 missense change detected in the family is mapped to the Kinesin motor catalytic domain and well conserved from *Homo sapiens* to *Drosophila melanogaster* with the exception of substitution by "K" which represents also a positive charge amino acid residue. **c** Native liver histology (HE stain) showed nodular cirrhotic liver tissue with scarce lymphomononuclear cell infiltration. The liver parenchyma showed obvious regenerative changes, without cholestasis. Higher magnification of the portal tract showed paucity of intrahepatic bile duct, with occasional lymphocytes infiltration. **d** Esophagogastroscopy showed the varices in the upper esophagus (**a** white arrow), lower esophagus (**b**, white arrow), gastric fundus (**c**, white arrow) and the varices after endoscopic variceal sclerotherapy (**d**, black arrow)

Search for novel monogenic candidates

In cases that no disease-causing variant was found in known or recently identified cholestasis related genes, we searched for novel monogenic candidates. We identified four participants with variants in what we ultimately considered to be candidate genes for cholestatic liver disease (Table 2). Two of them (*NCOA6* and *CCDC88B*) were affected by biallelic variants, one (*USP24*) by a de novo variant and one (*ATP11C*) by a hemizygous variant. All four candidate genes are highly expressed in hepatocytes or glandular cells (Supplementary Figure S4). Among the identified candidates of interest, the patients with variants in *NCOA6* and *ATP11C* recapitulate the cholestasis phenotype in mice models [9, 10].

High-level candidate genes

NCOA6 encodes a transcriptional coactivator that enhances the transcriptional activator functions of nuclear hormone receptors. In the family 53, we described a 7-month-old girl presented with neonate onset high GGT cholestasis, hepatosplenomegaly, developmental delay and growth delay. Her brain MRI showed the gyri are less numerous, dysplastic lateral ventricles and the sulci shallower less deep than normal (Fig. 4B). WES revealed compound heterozygous missense variants (c.1274A > G;p.Asn425Ser/c.1047G > C;p.Leu349Phe) in *NCOA6* gene. The variant c.1274A > G is absent in gnomAD and the variant c.1047G > C is present at a very low frequency of 0.000039 but never in homozygosity (Fig. 4A and Table 2). The persistent cholestasis was not responsive to the administration of UDCA until 9 months old (Supplementary Table S1). Overexpression of N-terminally Flag-tagged cDNA constructs modeling the WT and two variants in HEK293 cells showed that both aberrant alleles resulted in decreased protein expression (Fig. 4D). Previous study has shown that NCOA6 enables the activation of BSEP, NTCP, and MRP2 genes by nuclear receptors FXR/RXR [11]. To test whether the patient derived NCOA6 mutations affect the activation of the BSEP promoter activity. We transfected HEK293 cells with BSEP promoter and cotransfected with FXR in the presence of WT and mutant NCOA6full-length cDNA-encoding plasmids. As a result, significant decreased stimulation of BSEP promoter activity was seen in mutant NCOA6 groups (Fig. 4E).

ATP11C encodes a P4-type ATPase with aminophospholipid flippase activity at the hepatic canalicular membrane, which is essential for bile salt homeostasis and cause cholestatic liver disease if mutated in mice [12, 13]. In the family 56, a hemizygous truncating mutation (c.312_313insT;p.T105Yfs*8) in *ATP11C* was identified in a patient with agranulocytosis, recurrent acute liver failure and developmental delay (Fig. 5). The variant is never reported in gnomAD and predicted to cause loss of function, because the resulting mRNA transcript is likely to subjected nonsense-mediated decay (Table 2 and Fig. 5D). The patient is 4.5 years old currently under rehabilitation training with normal ALT/AST and TBIL.

Table 2 Cases with variants in candidate genes, along with available evidence from the literature to support candidacy

Patient ID	Age at onset	Gene symbol	Genotype	Segregation	gnomAD (hom/het / WT allele count)	PPH2	CADD	Mutation Taster	Gene Description	KO phenotype in mice	Supporting Evidence	Reference
53	1 month	*NCOA6*	c.1274A>G;p.Asn425Ser(Het)	Maternal	Never reported	0.15	17.4	DC	Nuclear receptor coactivator 6	Die from general growth retardation, developmental defects in heart, liver, brain and placenta	An epigenetic player involved in the activation of BSEP, NTCP and MRP2 gene by nuclear receptors FXR/RXR.	PMID: 21330447
			c.1047G>C;p.Leu349Phe(Het)	Paternal	0/11/282032	0.664	22.1	DC				
54	2 months	CCDC88B	c.3292C>T;p.Arg1098Trp(Het)	Maternal	0/14/151774	1	29.7	DC	Coiled-coil domain-containing protein 88B	ND	Known risk loci for primary sclerosing cholangitis identifies.	PMID: 27992413
			c.1307C>G;p.Ser436Cys(Het)	Paternal	0/125/187366	0.997	23.2	DC				
55	2 months	USP24	c.2282A>G;p.His761Arg(Het)	De novo	Never reported	0.92	21.6	DC	Ubiquitin carboxyl-terminal hydrolase 24	ND	USP24 has been shown on yeast two-hybrid assays to interact with USP53.	PMID: 23159851
56	1.5 years	ATP11C	c.312_313insT;p.Thr105Tyrfs*8 (Hemi)	Maternal	Never reported			DC	ATPase phospholipid transporting 11C	Conjugated hyperbilirubinemia, B-cell lymphopenia, hypercholanemia	A homolog of ATP8B1, catalyze the transport of phospholipids in biological membranes and essential for basolateral membrane localization of bile salt transport proteins.	PMID: 26926206

CADD Combined Annotation Dependent Depletion, *FXR* farnesoid X receptor, *gnomAD* genome aggregation database (https://gnomad.broadinstitute.org/), *Heimi* hemizygous, *Het* heterozygous, *KO* knockout, PPH2 score, PolyPhen-2 prediction score (0.0–1.0; i.e.; tolerated to deleterious; variants from 0.85 to 1 are more confidently predicted to be damaging) (http://genetics.bwh.harvard.edu/pph2/); Red and blue background represents deleterious prediction and likely benign prediction by the in silico algorithm, respectively. Black background represents deletion

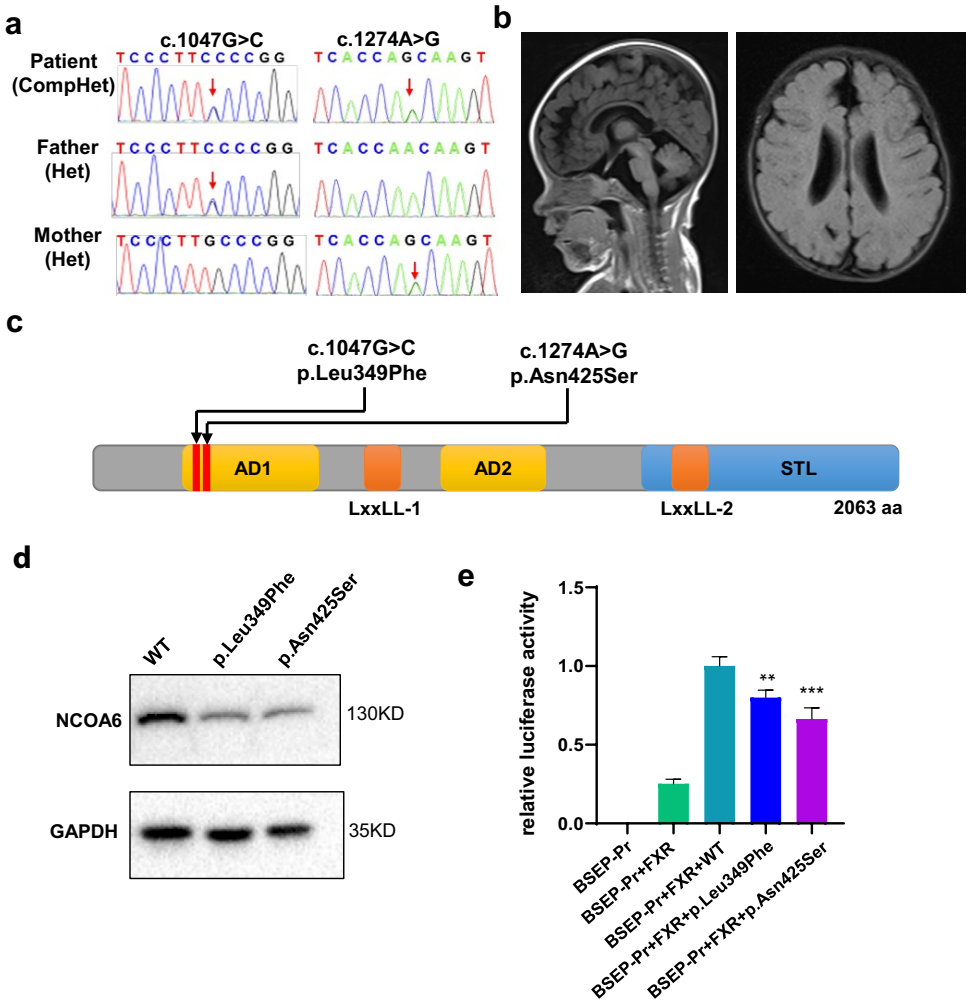

Fig. 4 Trio-based WES discovered compound heterozygous variants in *NCOA6* from a high GGT cholestasis patient. **a** Sequencing chromatograms showing the compound heterozygous variants c.1047G > C and c.1274A > G in the *NCOA6* gene of the *NCOA6*-related high GGT cholestasis family. **b** T1-weighted sagittal and T2-weighted axial images of the *NCOA6*-related patient shows the gyri are less numerous, dysplastic lateral ventricles and the sulci shallower less deep than normal. **c** *S*chematic representation of the protein showing the domain position of each of the mutations identified in this family. (AD, activation domain; LxxLL, LxxLL receptor interacting modifs; STL, serine, threonine and leucine rich region; red blocks, mutation sites). **d** Transient overexpression of N-terminally Flag-tagged cDNA constructs modeling the wild-type (WT) allele and two independent *NCOA6* mutations (p.Leu349Phe and p.Asn425Ser) in HEK293 cells. The two missense mutations resulted in decreased protein expression. **E** Overexpression of wild type and mutant *NCOA6* constructs, and FXR transactivation of BSEP promoter was monitored by luciferase activity. Significant decreased (**$p < 0.001$, ***$p < 0.0001$ compared with activity in cells with wild type *NCOA6* plasmid cotransfection) stimulation of promoter activity was seen with mutant *NCOA6* constructs

Suspected candidate genes

CCDC88B encodes Hook-related protein 3, a microtubule-binding protein involved in the physiological function of cilia [14]. In the family 54, compound heterozygous recessive missense variants (c.3292C > T; p.Arg1098Trp and c.1307C > G;p.Ser436Cys) in *CCDC88B* were found in a 2-month-old male patient with persistent cholestasis with high direct bilirubin, ALT, AST, and normal GGT. The two variants are both rare according to gnomAD and predicted to be pathogenic in silico (Table 2, Supplementary Figure S5). He continued to have low GGT cholestasis at the age of 6 months and then lost following up. Primary ciliopathy is a proven cause of primary sclerosing cholangitis, similar as DCDC2 deficiency, a known cause of neonatal sclerosing cholangitis which usually presenting as neonatal cholestasis [15]. Notably, *CCDC88B* is a known risk loci for primary sclerosing cholangitis confirmed in a GWAS study [16].

USP24 encodes ubiquitin carboxyl-terminal hydrolase 24, which has been shown on yeast two-hybrid assays to interact with *USP53* [17]. *USP53* has recently been confirmed to cause progressive intrahepatic cholestasis-7 with or without

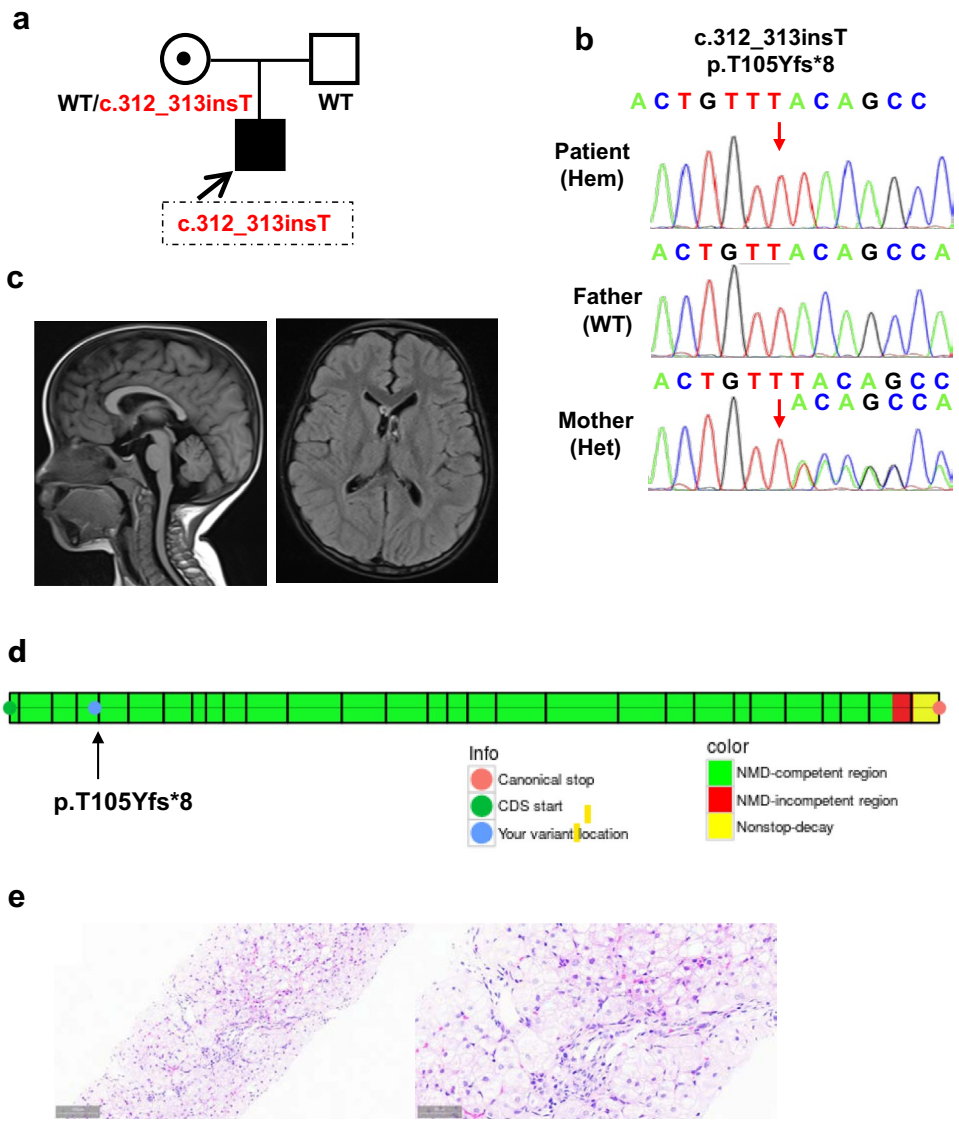

Fig. 5 Trio-based WES identified hemizygous variant in *ATP11C* in a patient with low GGT intrahepatic cholestasis. **a** Pedigrees of the family with *ATP11C*-related low gamma glutamyl transpeptidase (GGT) cholestasis. **b** Sequencing chromatograms showing the hemizygous variant c.312_313insT in the *ATP11C* gene of the *ATP11C*-related family. **c** T1-weighted sagittal and T2-weighted axial images of the *ATP11C*-related patient shows thin splenium of corpus callosum and slight fetal cerebral ventriculomegaly. **d** Schematic representation of the *ATP11C* gene showing the localization of truncating variant p.T105Yfs*8 that subjecting (NMD$^+$) to nonsense-mediated RNA decay (https://nmdpredictions.shinyapps.io/shiny/). The loss-of-function variant p.Gln169* detected in this study is shown in red. The region of ATP11C where truncating variants trigger NMD is indicated in green. **e** The liver biopsy performed at the age of 4.5 years demonstrating disrupted hepatic architecture, hepatocyte swelling and balloon degeneration, mild periportal fibrosis and lymphocytes inflammation

hearing loss (PFIC7, OMIM #619,658) [18]. In the family 55, we identified a de novo heterozygous variant in *USP24* in a 2-month-old male patient presenting with high GGT cholestasis, high total serum bile acids, direct bilirubin, ALT, AST and ALP. The de novo variant c.2282A > G;p.His761Arg is absent in gnomAD and local exome database and is predicted pathogenic by in silico tools(Table 2, Figure S5). The cholestasis was responsive to UDCA. The patient is currently 2 years old and the ALT/AST and BIL normalized with time but GGT continued to be elevated.

Discussion

Monogenic disorders are the second most common cause of cholestatic jaundice in the first months of life [1]. A precise genetic diagnosis of cholestatic jaundice patients can lead to more targeted treatment approaches [19], as well as help patients avoid unnecessary clinical investigations and guide recurrence risk estimation and genetic counseling. In a pediatric cohort, we identified disease-causing variants in 31% of participants with cholestatic jaundice through exome sequencing analysis with variant interpretation. This yield is similar to recent reports of NGS in Asian pediatric cholestasis cohorts [4, 20, 21]. The top six genes identified in definite diagnosed patients in this cohort were: *SLC25A13, JAG1, ABCC2, SLC10A1, ABCB11,* and *ABCB4* which was similar to cohorts from the Mongolian race but different from cohorts from the Caucasian race, suggesting that the disease spectrum of pediatric inherited cholestasis varies among different races [3–5, 20–22].

Several previous studies have suggested that low GGT is related to monogenic cholestasis and that patients with low GGT cholestasis should prioritize genetic testing [20,

22, 23]. However, in our cohort, the patients with solved causes had higher levels of GGT and lower levels of ALT and AST, suggesting that biochemical enzymes may not be reliable biomarkers of genetic cholestatic liver disease. In this study, the diagnostic rate of patients with extrahepatic findings was higher than that of those without extrahepatic manifestations (43.8% vs. 23.5%), indicating that multiple system involvement should be a red flag for genetic disorders in pediatric cholestasis patients.

With advancements in molecular biology, more and more monogenic disorders have been recently identified as the etiology of cholestatic liver diseases, such as *KIF12, PPM1F, USP53, LSR, WDR83OS, ZFYVE19, UNC45A, TTC26, ABCC12, SLC51A, SLC51B,* and *SEMA7A* [18, 24–29]. WES evaluates all single variants of exons, allowing for the data of the proband to be reanalyzed at any time. In this study, three patients were newly diagnosed through reanalysis, improving the diagnostic yield of the cohort.

In 2018, *USP53* was newly recognized as a gene that causes cholestasis, with deficiency in this gene also known as PFIC7 according to OMIM [18]. The *USP53* gene is located at 4q26, encodes inactive ubiquitin carboxyl-terminal hydrolase 53 which is a component of the tight junction complex [30]. To date, 34 cases have been reported, mainly presenting with low GGT cholestasis, itching, and some patients also suffering hearing loss [18, 31–33]. Our patient with USP53 deficiency had not received a molecular diagnosis in 2018 when she underwent WES, but exhibited low GGT cholestasis without deafness. Similar to other USP53 deficiency patients, her jaundice was relieved with UCDA. As some patients with USP53 deficiency developed splenomegaly with age [31, 33], further follow-up is required.

Through reanalyzing, a KIF12 deficiency patient was newly diagnosed. KIF12 deficiency also known as progressive intrahepatic cholestasis-8 according to OMIM [18], was first described in 2018. The *KIF12* gene is located at 9q32 and encodes Kinesin-like protein 12, which associates with the mitotic spindle and cleavage furrow [34]. To date, 13 cases with *KIF12* gene mutations in nine consanguineous families have been reported [18, 34, 35]. Our patient shared a similar clinical phenotype with other patients, characterized by high GGT neonatal cholestasis with rapid progression to liver fibrosis, and suffered from recurrent bleeding attacks due to portal hypertension and esophageal varices but without renal pelvic abnormalities [18]. The liver biopsies of our patients showed similar histopathology with most previously reported patients as hepatocellular and canalicular cholestasis, fibrosis with nodule formation, mixed portal inflammatory infiltrate, and bile duct loss. Together with our patient, five patients had living-related liver transplants due to liver failure [18, 34, 35].

In our cohort, one unsolved high GGT cholestasis patient showed slowly improved jaundice but stubborn skin lesions during follow-up. After re-phenotyping the patient and reanalyzing the WES data, we discovered that this patient had Hereditary fibrosing poikiloderma with tendon contractures, myopathy, and pulmonary fibrosis (POIKTMP) due to the de novo variant c.1883G > A(p.Ser628Asn) in the *FAM111B* gene. POIKTMP is an extremely rare syndromic form of autosomal dominant syndrome characterized by poikiloderma, hypo hidrosis with heat intolerance, mild lymphedema, chronic erythematous, sclerosis of the digits, mild palmoplantar keratoderma and uncommonly, liver impairment besides cholestasis [36]. The *FAM111B* gene is located at 11q12 and encodes Serine protease, whose function has not been established [37]. To date, 23 familial cases from 5 independent families and 13 sporadic cases have been reported [36–39]. Among the 36 patients, 13 showed liver impairment, including nine with high transaminases, four with cholestasis, and one who died of cirrhosis at the age of 17, indicating that POIKTMP is a new syndromic cholestasis. This patient benefited from the supplementary description of the phenotype and the reanalysis of WES data.

Beyond analyzing variants in newly published cholestasis-causing genes, WES has been shown to be a powerful tool for identifying monogenic causes of pediatric inherited cholestasis [18]. Our additional search for novel candidates in unsolved WES families revealed potential candidate genes (*NCOA6*, *CCDC88B*, *USP24* and *ATP11C*) involved in two low GGT and two low GGT cholestasis patients. Among these candidates, *NCOA6* and *ATP11C* are the most potential new monogenic causes based on the resembling cholestatic phenotypes in existing mice models. NCoA6-/- embryos die between 8.5 and 12.5 dpc due to general growth retardation and developmental defects in the heart, liver, brain, and placenta [9, 40]. Several studies have elucidated the role of *NCOA6* in bile acid homeostasis [11, 41]. An in vivo study from Xu et. al demonstrated that mutation of the NCoA6 LXXLL-2 motif in mice resulted in high cholesterol-diet-induced hepatic cholesterosis and hypercholesterolemia [41]. Besides, Suchy et.al have shown that the NCOA6 serve as an epigenetic player which is involved in the activation of BSEP, NTCP, and MRP2 genes by nuclear receptors FXR/RXR [11]. Additionally, NCOA6 is downregulated in cholestasis of a post-common bile duct ligation (CBDL) model in mice [11]. Based on these findings, we suggest that loss of function variants in *NCOA6* may be responsible for cholestatic liver diseases in humans. ATP11C is a homolog of ATP8B1 and belongs to the transport of phospholipids in biological membranes [10]. In mice, ATP11C localized to the basolateral membrane of central hepatocytes in the liver lobule may act as a gatekeeper to prevent hepatic bile salt overload [13]. Mice deficient in *ATP11C* are characterized by conjugated hyperbilirubinemia, hyperchloremia, and hemolytic anemia [12, 13, 42]. The liver and hematological symptoms of a patient with a truncating variant

(c.312_313insT;p.T105Yfs*8) in the *ATP11C* gene highly recapitulate the phenotype of atp11c mutant mice, strongly suggesting that the loss of function variant is the etiology of the case.

Conclusion

In our study of a single pediatric cholestasis cohort, we were able to identify monogenic variants in 22 known human intrahepatic cholestasis or phenocopy genes, providing an explanation for up to 31% of the intrahepatic cholestasis patients. By investigating newly identified genes associated with cholestasis, we were able to provide a molecular diagnosis to three additional patients. These findings highlight the importance of regularly re-evaluating existing WES data to improve diagnostic yield for patients with unknown etiology. Moreover, our WES full evaluation has led us to identify *NCOA6, CCDC88B, USP24,* and *ATP11C* as new potential monogenic causes for genetically undiagnosed cases of cholestasis. However, we must note that although *NCOA6* and *ATP11C* resemble cholestatic phenotypes in existing mouse models and have relevant biological functions, confirmation through additional cases with an identical cholestasis phenotype is needed.

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Declarations

Conflict of interest Yucan Zheng, Hongmei Guo, Leilei Chen, Weixia Cheng, Kunlong Yan, Zhihua Zhang, Mei Li, Yu Jin, Guorui Hu, Chunli Wang, Chunlei Zhou, Wei Zhou, Zhanjun Jia, Bixia Zheng, and Zhifeng Liu disclose no conflicts.

Ethical approval The study was approved by the Ethics Committee of Children's Hospital of Nanjing Medical University (no.202012090-1). A written informed consent was obtained from all patients or their guardians.

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