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ORIGINAL ARTICLE

The utility of hierarchical genetic testing in paediatric liver disease

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Abstract

Background & Aims: Genetic factors underlie a substantial proportion of paediatric liver diseases. Hereditary liver diseases have considerable genetic heterogeneity and variable clinical manifestations, which bring great challenges to clinical and molecular diagnoses. In this study, we investigated a group of paediatric patients with varying degrees of liver dysfunction using a hierarchical genetic testing strategy.

Methods: We first applied a panel encompassing 166 known causal genes of liver disease. We then used exome sequencing (ES) in those patients whose cases remained undiagnosed to identify the genetic aetiology of their symptoms.

Results: In total, we enrolled 131 unrelated paediatric patients with liver disease of Chinese Han ethnicity. We first applied targeted gene sequencing of 166 genes to all patients and yielded a diagnostic rate of 35.9% (47 of 131). Eighty-four patients who remained undiagnosed after target gene sequencing were subjected to ES. As a result, eight (8/84, 9.5%) of them obtained molecular diagnoses, including four patients suspected of abnormal bilirubin metabolism and four idiopathic cases. Non-typical genetic findings, including digenic inheritance and dual molecular diagnosis, were also identified. Through a comprehensive assessment of novel candidate variants

Abbreviations: ACMG/AMP, American College of Medical Genetics and Genomics/Association for Molecular Pathology; ACMG/ClinGen, American College of Medical Genetics and Genomics and the Clinical Genome Resource; AF, allele frequency; CNV, copy number variants; ComHet, Compound heterozygous; ES, exome sequencing; F, Female; gnomAD, Genome Aggregation Database; Het, Heterozygous; Hom, Homozygous; M, male; N/A, not applicable; SNV, single nucleotide variant.

Fuchuan Wang, Yaqi Li, and Sen Zhao contributed equally to this study.

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of uncertain disease association, 11 patients of the remaining undiagnosed patients were able to obtain likely molecular diagnoses.

Conclusions: Our study presents evidence for the diagnostic utility of sequential genetic testing in a cohort of patients with paediatric liver disease. Our findings expand the understanding of the phenotypic and mutational spectrum underlying this heterogeneous group of diseases.

KEYWORDS
genetic testing, liver disease, molecular diagnosis, novel variants

1 | INTRODUCTION

Paediatric liver diseases cause approximately 15 000 hospitalizations costing a total of $350 million every year in the USA,[1] and have necessitated 12 046 liver transplants in European children from 1988 to 2015,[2] with 17% metabolic liver diseases and 49% cholestatic congestion. Hereditary disorders are important causes of paediatric liver diseases, comprised of a variety of Mendelian syndromes, including Wilson disease, glycogen storage disease, hereditary hemochromatosis, alpha-1 antitrypsin deficiency, and tyrosinemia type 1.[3–5] These diseases are often caused by genetic variants affecting genes essential for critical metabolic pathways.

With the development of next-generation sequencing, genetic testing has emerged as an efficient tool for the diagnosis of many hereditary diseases, including those affecting the liver. For patients with clinically suspected genetic disorders, targeted sequencing of candidate genes has demonstrated a considerable diagnostic utility, as exemplified in Wilson disease,[6] hereditary tyrosinemia type 1,[7] and hereditary hemochromatosis.[8]

Nevertheless, in clinical practice, hereditary liver diseases have considerable genetic heterogeneity and variable clinical manifestations, which bring challenges to the design of appropriate gene panels for sequencing.[9–17] Exome sequencing (ES) represents a relatively more comprehensive genetic testing method. Although more costly than panel sequencing, ES has been shown to substantially increase the diagnostic rate in cohorts of various metabolic diseases and developmental disorders. ES also enables the classification of more complicated conditions, e.g., dual diagnosis, where two molecular diagnoses are simultaneously observed in a single individual, resulting in blended phenotypes.[18]

In this study, we investigated a group of paediatric patients with liver disease. We first applied panel sequencing encompassing 166 causal genes of liver diseases. Then we used ES in the remained cases without a diagnosis from panel sequencing, which allowed us to identify more disease-associated variants.

2 | PATIENTS AND METHODS

2.1 | Patients

Paediatric patients (age ≤18) with paediatric liver disease were recruited from the Fifth Medical Center of Chinese PLA General Hospital from January 2015 to May 2018.

Patients with either of the following conditions were included in our study: (1) abnormal liver function examination, i.e., exceeding the 2× upper limit of normal (ULN) range in alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase, total bile acid (TBA), total bilirubin or direct bilirubin or (2) abnormal findings in imaging, such as cirrhosis or hepatic fibrosis.

The exclusion criteria for our study were as follows: (1) patients with viral hepatitis (i.e., hepatitis A virus, hepatitis B virus, hepatitis C virus, Cytomegalovirus, or Epstein–Barr virus); (2) patients with autoimmune hepatitis; (3) patients with a definitive history of drug and toxin-related liver disease; (4) patients with diseases primarily affecting other systems; (5) patients meeting the diagnostic scoring standards for Wilson disease; (6) patients with hepatic neoplasm; (7) patients with a definitive history of abdominal trauma.This study was approved by the Research Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital (IRB ID: 2020078D). Consent forms were obtained from all patients or guardians.

2.2 | Sequencing and bioinformatic analysis

The gene panel used for targeted gene sequencing of liver disease includes 166 causal genes for 223 monogenic disorders, which are divided into 12 categories (Table S1). ES was performed on probands in cases where the targeted panel sequencing failed to identify any pathogenic variants. In this process, genomic DNA extracted from peripheral blood was prepared into Illumina paired-end libraries, and then subjected to exome capture using xGEN targeted capture kit (IDT) or All Exon V6 (Agilent), depending on

the time of enrollment in our cohort, followed by sequencing on the Illumina X-TEN platform.

Variant-calling and annotation were performed by the in-house developed Peking Union Medical College Hospital Pipeline (PUMP).[19] Single-nucleotide variants and internal duplications and/or deletions (indels) were called using the HaplotypeCaller of the Genome Analysis Toolkit (GATK), version 3.4.0.

Population allele frequency (AF) of each variant was obtained from the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) and an in-house database of 4983 exomes. Combined annotation dependent depletion (CADD) was used for in silico prediction of variant deleteriousness.

2.3 | Variant interpretation

The protocol for interpretation of single nucleotide variants (SNVs) and indels was adapted from the American College of Medical Genetics and Genomics (ACMG) guidelines.[20] For dominant disorders, one pathogenic or likely pathogenic variant in a gene that can account for a patient's phenotype(s) is required for a molecular diagnosis. For the molecular diagnosis of recessive disorders, bi-allelic pathogenic or likely pathogenic variants are required. In this study, we also reported novel clinically relevant candidate variants associated with observed phenotypes, particularly in cases where patients carry one pathogenic variant and one variant of uncertain significance (VUS) in recessive genes. Intragenic copy number variants (CNV) in suspected genes were detected by manual review of bam files using the integrative genomics viewer (IGV).[21]

2.4 | PCR and Sanger sequencing

When a suspected variant was identified in the proband, Sanger sequencing for SNV or quantitative PCR(qPCR) for CNV was performed on family trios and other affected family members for validation. Additionally, we conducted PCR on four patients and one control to verify a long-range insertion to intron 3 of *SLCO1B3* found through ES. For PCR, we designed a forward primer complementary to the insertion's terminal sequence (5′-CTAAGCAAGCCTGGGCAATG−3′) and a reverse primer complementary to the flanking inversed-exon 4 sequence (5′-TCAATGGAACATCACCTGAGA-3′). PCR products were then sequenced by Sanger sequencing.

2.5 | In-house exome database

An in-house database of 4983 Chinese individuals who underwent ES was collected through the Deciphering disorders Involving Scoliosis and COmorbidities (DISCO) study (http://www.discostudy.org/). Exome data were processed and joint-called using the Peking Union Medical College hospital Pipeline (PUMP).[19]

2.6 | Cost-effectiveness analysis

A simulation-based method was used to compare the cost-effectiveness of the hierarchical strategy implemented in this study and a counterfactual first-line ES strategy, in which all patients directly undergo diagnostic ES without prior tests (Figure S1). The detailed cost and diagnostic time for each strategy were consistent with the Chinese Medical Service Price Regulations (Table S2). Chinese Yuan (CNY) was converted into American Dollars (USD) based on the exchange rate of 0.155 on 31 July 2021. We calculated the mean cost differences and the mean diagnostic time differences with 95% parametric confidence intervals for effectiveness comparison for the whole cohort and clinical subgroups with $n > 10$. We then performed $n = 500$ bootstraps to test the robustness of the parametric assumptions. Each bootstrap included $n = 500$ individuals randomly selected from the cohort ($n = 131$).

2.7 | Statistics

We used the Pearson chi-squared analysis module in IBM SPSS Statistics 26 to compare allele counts between different population databases (i.e., all populations in the GnomAD exome database and the Chinese population represented by our in-house database of 4983 individuals). We performed Fisher's exact test in the event of an expected count below 5 in 2×2 contingency table. Differences with a $p < 0.05$ were reported as significant.

3 | MAIN OUTCOMES AND MEASURES

3.1 | Cohort demographics and molecular diagnostic yield

After excluding patients who were diagnosed based on clinical examinations (Table S3), we enrolled 131 unrelated paediatric patients with unexplained liver dysfunction (Figure 1, Table 1). The mean age of symptom onset is 5.7 ± 5.3 years, and 38.1% of the patients are female. While 80 out of 131 patients were clinically suspected of having a genetic disorder such as Wilson disease or glycogen storage disease, the rest of the patients (51/131, 38.9%) were not specifically likely to have a specific genetic disorder, and these were considered as idiopathic cases.

We first applied targeted gene sequencing, surveying 166 genes to all 131 patients, and yielded a diagnostic rate of 47 out of 131 (35.1%) (Figure 2, Table S4, Table 3). Most patients clinically suspected of having Wilson disease (11 of 15) or glycogen storage disease (19 of 23) obtained genetic diagnoses. Fourteen cases out of 19 patients diagnosed with glycogen storage disease were further subclassified. Most of them (8 of 14) were found to be affected by glycogen storage disease IXa. Notably, seven out of 51 (14%) patients in the idiopathic group were also molecularly diagnosed through the targeted gene sequencing (Figure 2).

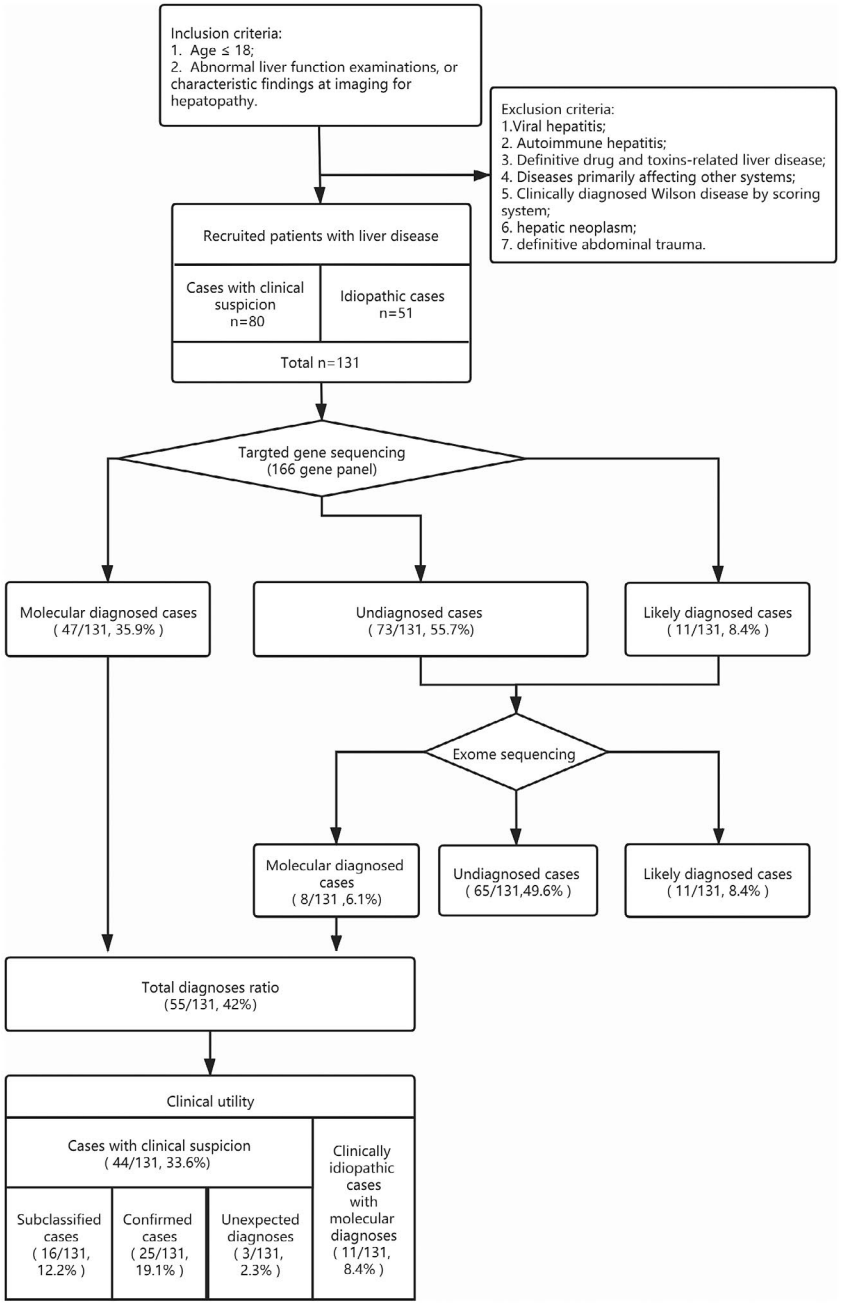

FIGURE 1 Diagram of the hierarchical genetic testing workflow

Pathogenic CNVs identified by manual review of bam files were revealed in three patients (Table 3). A homozygous exon 1 loss in *PHKA2* enabled the diagnosis of glycogen storage disease, type IXa1, of one idiopathic patient. A heterozygous deletion, involving the entire exon 1 to exon 10 of the *OTC* gene, confirmed the clinically suspected diagnosis with urea cycle disorder in another patient. One male patient (DISCO-H16070594) simultaneously harboured an exon 51 deletion in *DMD* and a pathogenic mutation in *ATP7B*. The details of the dually diagnosed patient (DISCO-H16070594) were provided in the later section.

Three patients received molecular diagnoses unexpected from the suspected clinical diagnoses. They presented with atypical phenotypes in the context of their underlying genetic disorders (Table S5). Patient DISCO-H17044399, molecularly diagnosed with Wilson disease, presented high urinary copper concentration (172.7 μg per 24 h, higher than 2× upper limit of normal [ULN]), but normal serum ceruloplasmin concentration. Patient DISCO-H171110226, molecularly diagnosed with Polycystic kidney disease 4, presented hepatic fibrosis with relatively low serum ceruloplasmin concentration. Another patient, DISCO-H16050225, molecularly diagnosed with Alagille syndrome 1, had hyperlipemia and foam cells in his bone marrow biopsy, which is frequently seen in lipid metabolic disorders (e.g., Niemann-Pick disease).[22]

Eighty-four patients who remained undiagnosed after target gene sequencing were subsequently subjected to ES, and eight patients obtained a molecular diagnosis through ES (Figure 2, Table 2), including four patients out of nine that were initially diagnosed to

TABLE 1 Cohort demographics and molecular diagnostic yield

Clinical suspicion	No. of cases	Female, no.	Age of presentation, mean (SD)	Diagnostic rate by PANEL	Additional diagnostic rate by ES	Overall diagnostic rate
Idiopathic	51	19/51	5.1 (5.4)	7 (14%)	4 (8%)	11 (22%)
Glycogen storage disease	23	9/23	3.6 (3.5)	19 (83%)	0 (0%)	19 (83%)
Wilson disease	15	4/15	7.1 (2.6)	11 (73%)	0 (0%)	11 (73%)
PFIC	11	4/11	8.1 (6.7)	0 (0%)	0 (0%)	0 (0%)
Abnormal bilirubin metabolism	9	4/9	8.6 (6.6)	0 (0%)	4 (44%)	4 (44%)
Alagille syndrome	4	3/4	5.5 (7.1)	2 (50%)	0 (0%)	2 (50%)
Gilbert syndrome	4	1/4	5.8 (7.5)	2 (50%)	0 (0%)	2 (50%)
Urea cycle disorder	4	4/4	3.1 (2.0)	4 (100%)	0 (0%)	4 (100%)
Congenital hepatitis fibrosis	3	1/3	14.3 (3.1)	0 (0%)	0 (0%)	0 (0%)
Niemann–Pick Disease	2	1/2	8.5 (4.9)	1 (50%)	0 (0%)	1 (50%)
Other	5	0/5	3.3 (5.0)	1 (20%)	0 (0%)	1 (20%)
Overall	131	50/131	5.7 (5.3)	47 (36%)	8 (6%)	55 (42%)

PFIC: Progressive familial intrahepatic cholestasis; Other: Hemochromatosis (one case), Mitochondrial DNA depletion syndrome, hepatic (one case), Citrin deficiency (one case), Cystic fibrosis (one case), Maple syrup urine disease (one case).

have abnormal bilirubin metabolism (44%), and four patients of the 51 that had idiopathic liver diseases (8%).

These four patients presenting with abnormal bilirubin metabolism were all diagnosed with Rotor syndrome. Rotor syndrome is a digenic-recessive bilirubin metabolism disorder caused by bi-allelic loss-of-function variants in both *SLCO1B1* and *SLCO1B3*, which encode for the protein OATP1B1 and OATP1B3, the main transporters for conjugated bilirubin uptake in hepatocytes.[23] In *SLCO1B1*, a homozygous stop-gain variant c.1738C > T (p.R580*) was found in all four patients. In *SCLO1B3*, we identified a long-interspersed element (LINE-1, L1) insertion in intron 3 and the consequent inversion of exon 4 (NM_001349920.2), which has been previously reported in Chinese patients with Rotor syndrome (Figure 3).[24] These findings highlight the importance of a comprehensive analysis of multigenetic mutations and recurrent structure variations in the bilirubin metabolism pathway.

The diagnoses of four idiopathic patients were comprised of one case of familial hypercholesterolemia (MIM: 143890), one case of Schwachman-Diamond syndrome (MIM: 260400), and two cases of transient infantile hypertriglyceridemia (MIM: 614480). The panel sequencing did not cover these genetic disorders due to the non-typical hepatic presentation (fatty liver cirrhosis in two patients with transient infantile hypertriglyceridemia caused by *GPD1*[25] and elevated ALT/AST/TBA in the patient with Schwachman-Diamond syndrome caused by *SBDS*[26]), or lack of updated inclusions based on associations described in recent literature (liver cirrhosis in the patient with familial hypercholesterolemia caused by *LDLR* [27]).

3.2 | Dual molecular diagnoses

One male patient (DISCO-H16070594) from a consanguineous family (Figure S2) had a homozygous c.2333G > T (p.Arg778Leu) pathogenic variant in the *ATP7B* gene (Table S5) and a hemizygous pathogenic CNV (exon 51 deletion) in *DMD* (Table 3), resulting in a dual molecular diagnosis of Wilson disease and Duchenne muscular dystrophy. The mutation, C.2333G > T (p.Arg778Leu) of *ATP7B* was reported in more than five previous cases of Wilson's disease,[28–30] and was shown to cause a reduction in ATP7B protein expression and copper export capacity.[31] The deletion of exon 51 in *DMD* also has been reported in more than five patients affected by *DMD*,[32–35] and putatively led to LoF of the DMD protein. The patient's serum aminotransferase was self-reported to be elevated during the past 3 years, and his liver biopsy showed the features of chronic hepatitis (Figure S3). Copper staining of the liver was positive; his urinary copper was 105.8 μg per 24 h (higher than 2× ULN). He also had low serum ceruloplasmin (0.02 g/L). Additionally, muscle magnetic resonance imaging showed fatty infiltration and multiple edemas in the bilateral gluteus maximus and thigh muscle. Blood tests showed high serum creatine kinase (7711 U/L), high ALT (429 U/L) and high AST (356 U/L). Taken together, the dual molecular diagnosis in this patient resulted in a blended phenotype, emphasizing the importance of genetic testing in diagnosing complex liver disorders.

3.3 | Cost-effectiveness of the hierarchical genetic testing strategy

To evaluate whether this hierarchical strategy is superior to the direct ES strategy in our paediatric liver disease clinical setting, we performed a cost-effectiveness analysis based on our realistic unexplained-liver-dysfunction cohort ($n = 131$). As a result, we found that the hierarchical strategy could averagely save 2.6 days (95%CI: −0.5 days to 5.8 days) in diagnostic time per patient, but needed an extra expense of $149.8 (95%CI: $61.6–$238.0), compared with the direct ES strategy (Table 4, Figure S4). In subgroup analysis, we

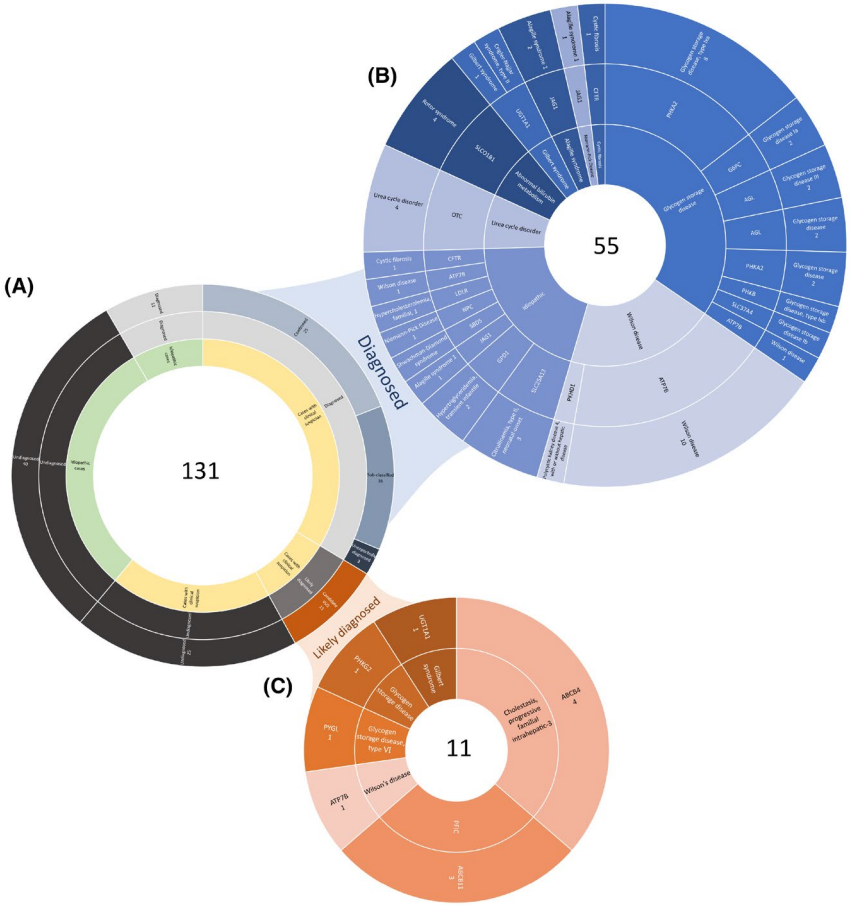

FIGURE 2 The clinical utility of genetic testing in identifying hereditary disorders. (a) The inner ring: suspected clinical diagnoses or idiopathic cases. The second-layer ring: the status of the molecular diagnosis. The third layer: "Confirmed" means the molecular diagnosis supports the suspected clinical diagnosis; "Sub-classified" cases were molecularly diagnosed in a specific subtype; "Unexpected diagnosed" means the molecular diagnosis deviated from the suspected clinical diagnosis; "Candidate VUS", refers to the potential contributive variant of uncertain significance (VUS) in patients with relevant phenotypes. (b) Diagnosed cases spectrum. Inner ring: suspected clinical diagnosis. Middle ring: pathogenic gene. The dually diagnosed patient (DISCO-H17033240) is counted in the Wilson disease group. (c) Likely diagnosed cases spectrum. Inner ring: clinical suspicion (also the likely-diagnosed genetic disorders), Middle ring: candidate gene. The numbers in the circle centres of (a), (b) and (c) are the total case numbers. The numbers in the third-layer rings in (a), (b) and (c) are the numbers of cases of each subgroup

found that the hierarchical strategy could save money and time in clinical entities with a relatively high panel-based diagnostic rate, i.e. Glycogen storage disease and Wilson disease. However, for the idiopathic group where panel sequencing had a low diagnostic rate, direct ES Strategy showed better effectiveness in both time and cost (Table 4).

3.4 | Potential novel candidate variants

For patients highly suspected of having a specific disease but remained undiagnosed after genetic testing, we prioritized 19 novel clinically relevant candidate variants in 11 patients (Figure 2, Table S6). These candidate variants included 17 missense variants, one splice variant, and one promoter variant. All variants were absent from public databases. As a result, these patients achieved likely molecular diagnoses for several recessive disorders, including progressive familial intrahepatic cholestasis (PFIC) (n = 7), Gilbert syndrome (n = 1), glycogen storage disease (n = 2) and Wilson disease (n = 1). Among the 11 patients, five patients had one novel candidate variant *in trans* with a pathogenic/likely pathogenic variant, while six patients had one candidate variant in each allele. Two patients (DISCO-H17087497 and DISCO-H16010413), who received likely diagnoses of PFIC, had been previously diagnosed with Wilson disease, due to abnormally high urinary copper, and had been taking copper-chelating medications. After the likely molecular diagnosis of PFIC, they suspended the anti-copper treatments and began symptomatic treatment. Another patient (DISCO-H17012450) had high urinary copper (1× upper limit of normal [ULN]) and low ceruloplasmin (0.03 g/L), scoring 3 points according to Leipzig scoring system ("Diagnosis possible, more tests needed").[36] After genetic testing, the novel candidate variants in *ATP7B* suggested a likely diagnosis of Wilson disease. This patient began copper-chelating therapy, which successfully alleviated his disease symptoms.

TABLE 2 Causal variants identified by exome sequencing

Patient ID	Gender	Age of presentation (y)	Clinical suspicion	Gene	Zygosity	Reference sequence	Genomic position	cDNA change	Protein change	Mutation type	Molecular diagnosis	MIM ID	Inheritance	Origin	ACMG/AMP category
DISCO-H16040350	F	3	Idiopathic	*LDLR*	Het	NM_000527.4	chr19:11231108	c.2050G > A	p.Ala684Thr	Missense variant	Hypercholesterolemia, familial, 1	143890	AD	Inherited Heterozygous	Pathogenic
DISCO-H1800155301	M	1	Idiopathic	*SBDS*	Het	NM_016038.2	chr7:66459197	c.258 + 2 T > C	N/A	Splice donor variant	Shwachman-Diamond syndrome	260400	AR	ComHet	Pathogenic
DISCO-H1800155301	M	1	Idiopathic	*SBDS*	Het	NM_016038.2	chr7:66459273	c.184A > T	p.Lys62Ter	Stop gained	Shwachman-Diamond syndrome	260400	AR	ComHet	Pathogenic
DISCO-H1801274901	M	0.1	Idiopathic	*GPD1*	Het	NM_005276.3	chr12:50499329	c.220-2A>G	N/A	Splice acceptor variant	Hypertriglyceridemia, transient infantile	614480	AR	ComHet	Pathogenic
DISCO-H1801274901	M	0.1	Idiopathic	*GPD1*	Het	NM_005276.3	chr12:50500108	c.398C > T	p.Ser133Leu	Missense variant	Hypertriglyceridemia, transient infantile	614480	AR	ComHet	Likely pathogenic
DISCO-H1801373201	F	1	Idiopathic	*GPD1*	Het	NM_005276.3	chr12:50499329	c.220-2A > G	N/A	Splice acceptor variant	Hypertriglyceridemia, transient infantile	614480	AR	ComHet	Pathogenic
DISCO-H1801373201	F	1	Idiopathic	*GPD1*	Het	NM_005276.3	chr12:50501372	c.635G > T	p.Gly212Val	Missense variant	Hypertriglyceridemia, transient infantile	614480	AR	ComHet	Likely pathogenic
DISCO-H17098039	M	8	Abnormal bilirubin metabolism	*SLCO1B1*	Hom	NM_006446.4	chr12:21375289	c.1738C > T	p.Arg580Ter	Stop gained	Rotor syndrome	237450	DR	Homozygous	Pathogenic
DISCO-H1802175201	F	1	Abnormal bilirubin metabolism	*SLCO1B1*	Hom	NM_006446.4	chr12:21375289	c.1738C > T	p.Arg580Ter	Stop gained	Rotor syndrome	237450	DR	Homozygous	Pathogenic
DISCO-H1800015101	F	24	Abnormal bilirubin metabolism	*SLCO1B1*	Hom	NM_006446.4	chr12:21375289	c.1738C > T	p.Arg580Ter	Stop gained	Rotor syndrome	237450	DR	Homozygous	Pathogenic
DISCO-H17087795	M	2	Abnormal bilirubin metabolism	*SLCO1B1*	Hom	NM_006446.4	chr12:21375289	c.1738C > T	p.Arg580Ter	Stop gained	Rotor syndrome	237450	DR	Homozygous	Pathogenic

Abbreviations: DR: Digenetic recessive inheritance of *SLC1B1* and *SLC1B3* genes, N/A: Not applicable.

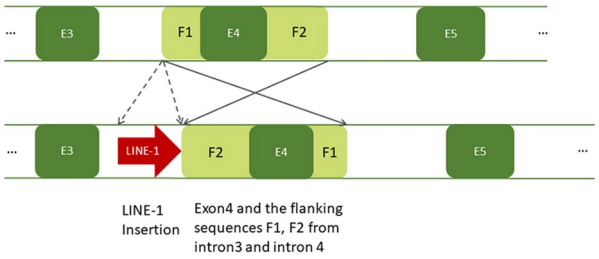

FIGURE 3 LINE-1 insertion and the consequent sequence inversion in *SLCO1B3*. Exons are marked as E. Flanking sequences are marked as F1 and F2. The insertion of LINE-1 resulted in the reversion of F1, E4 and F2

3.5 | Identification of recessive alleles enriched in the Chinese population

We identified eight recurrent recessive alleles with extremely low AFs in population databases (GnomAD minor allele frequency [MAF]<0.0001). By inspecting their AFs using our in-house database of the Chinese population, we found that five variants were significantly enriched in our in-house database compared to the gnomAD database representing mixed populations (Table 5), suggesting that these variants are enriched in the Chinese population and warrant attention in carrier screening and genetic counselling.

4 | DISCUSSION

In this study, we report findings of panel sequencing and ES in a cohort of paediatric patients with liver disease. The hierarchical strategy of using gene panel sequencing supplemented by ES allowed us to achieve molecular diagnoses in 55 patients and likely molecular diagnoses in 11 patients.

As a screening test, panel sequencing provided molecular diagnostics to 47/131(36%) in all the patients and 7/51(14%) in the idiopathic cases. The diagnostic rate of panel sequencing on idiopathic patients is consistent with prior reports: Chen et al. applied a panel with 52 genes to 38 unspecified infant cholestasis, and the diagnostic rate is 5/38(13.1%).[37] Santos Silva et al. used evoluting gene panels (from 54 genes to 95 genes) to 13 paediatric patients, and the diagnostic rate is 2/13(15.4%).[38] Moreover, Lipiński et al. implemented a panel with 1000 clinically relevant genes, and the sequencing on patients with chronic cholestatic liver disease of an unknown aetiology led to a molecular diagnosis in 15/22 patients (68%).[39]

Although most of the patients were successfully diagnosed using the gene panel, ES played an essential role in identifying causal variants in etiologically heterogeneous conditions such as cholestasis. The diagnostic utility of ES was also corroborated in a recent study on adult idiopathic liver disease, where 5 out of 19 cases of chronic liver disease with unknown aetiology were solved by ES.[40] Taken together, ES can be utilized as a last resort for patients with unexplained liver diseases.

Among the 55 patients with molecular diagnoses, genetic testing confirmed or specified the initially suspected diagnoses of 41 patients. The remaining 14 patients who received successful molecular diagnoses were initially idiopathic or received unexpected diagnoses. They demonstrated the variable expressivity and incomplete penetrance underlying these Mendelian disorders. With the extensive application of genetic testing in clinical practice, we are uncovering an increasing number of such ambiguous conditions. Therefore, a genotype-first approach focusing on pathogenic variants over patients' phenotypes has been proposed.[41,42] This approach is able to identify patients with pathogenic or likely pathogenic variants, despite having only partial phenotypic matches to known genetic disorders.[42] However, such individuals may not fulfil the full clinical diagnostic criteria and require careful evaluation and continued monitoring for potential late-onset presentations.

Our data revealed a diverse variety of liver disease-associated variants. In addition to SNVs and indels, we also identified short tandem repeats, intragenic CNVs, and exon inversion caused by transposon insertion. One of the likely diagnosed patients (DISCO-H17076878) had a heterozygous dinucleotide-repeat polymorphism, (TA)7/(TA)6, in TATA box of *UGT1A1* promoter. While A(TA)6TAA is the major allele, $A(TA)_7TAA$ in TATA box is the second common mutation, and homozygosity for $A(TA)_7TAA$ has been shown to cause lower binding affinity of TATA-binding protein both in patients[43] and in vitro experiments.[44] Although TA 6/7 alone may not cause significantly lower *UGT1A1* mRNA like pathogenic TA 7/7, the combination of TA 6/7 and another single nucleotide polymorphism (SNP) (e.g., −3279 T > G) of *UGT1A1* can lead to significantly lower *UGT1A1* mRNA transcription.[45] One of our patients (NG17076878) provides clinical evidence for the pathogenicity of combined variations in the non-exome region of *UGT1A1*.

Another atypical variant type identified in our study is the transposon insertion IVS16ins3kb in *SLC25A13*, a frequently reported pathogenic insertion variation in Chinese patients with citrin deficiency. A representative Chinese cohort study showed that IVS16ins3kb was found in 10.04% of the 522 alleles in all patients molecularly diagnosed with citrin deficiency. The variant frequency is significantly higher in the north and the border region.[46] Therefore, tandem repeats and structural variations should not be overlooked when analysing sequencing data.

In addition to pathogenic/likely pathogenic variants, we also selected 19 clinically relevant candidate variants and established likely molecular diagnoses in 11 patients with highly indicative clinical presentation. Patients' clinical management is challenging when facing a likely molecular diagnosis with novel disease-associated variants. The strategic decision should be made based on the medical record, family history, physical examinations, and genetic testing results.[47] In vitro functional studies have also proved to be an efficient tool to evaluate the pathogenicity of novel disease-associated variant,[48] which may be applied in the clinical context after rigorous validation.

Five recessive alleles were calculated to be enriched in the Chinese population. Four *ATP7B* variants (c.2621C > T, c.2333G > T, c.2755C > G, c.2975C > T) were consistent with the previous study in the Chinses Han population.[49] The *AGL* variant, c.1735 + 1G > T, was previously supposed to be prevalent in Chinese,[50] for which we

TABLE 3 Intragenic CNVs identified by manual review of read depth

Patient ID	Gender	Age of presentation	Clinical suspicion	Gene	Zygosity	Reference sequence	CNV region	CNV type	Molecular diagnosis	MIM ID	Inheritance	Origin	ACMG/ Clingen category
DISCO-H1801573901	M	0.58	Idiopathic	*PHKA2*	Hom	NM_000292.2	exon 1	loss	Glycogen storage disease, type Ixa1	306000	Autosomal recessive	Hemizygous	Pathogenic
DISCO-H16070594	M	7	Wilson disease	*DMD*	Hom	NM_004006.2	exon 51	loss	Wilson disease	277900	X-linked recessive	Hemizygous	Pathogenic
DISCO-H1801551701	F	2	Urea cycle disorder	*OTC*	Het	NM_000531.6	whole gene	loss	Urea cycle disorder	311250	X-linked recessive	Unknown	Pathogenic

TABLE 4 Cost-effectiveness analysis of the cohort and clinical subgroups

Clinical Suspicion	Strategy	Total cost (USD)	Total diagnostic time (d)	Total number of patients	Number of patients who underwent ES	Average cost per patient (95% CI) (USD)	Average cost difference (95% CI) (USD)	Average diagnostic time per patient (95% CI) (d)	Average diagnostic time difference (95% CI) (d)
Total	Hierarchical strategy	173376.7	6079	131	84	1323.5 [1241.0, 1406.0]		46.4 [44.1, 48.7]	
	ES strategy	153754.7	6424	131	131	1173.7 [1165.8, 1181.6]	149.8 [61.6, 238.0]	49.0 [47.6, 50.5]	−2.6 [−5.8, 0.5]
Glycogen storage disease	Hierarchical strategy	19778.8	810	23	4	859.9 [697.6, 1022.3]		35.2 [30.1, 40.4]	
	ES strategy	27828.8	1279	23	23	1209.9 [1196.9, 1223.0]	−350.0 [−519.4, −180.6]	55.6 [53.0, 58.2]	−20.4 [−26.4, −14.4]
Wilson disease	Hierarchical strategy	14277.3	563	15	4	951.8 [720.1, 1183.5]		37.5 [30.9, 44.1]	
	ES strategy	18039.3	816	15	15	1202.6 [1180.0, 1225.3]	−250.8 [−498.5, −3.1]	54.4 [50.0, 58.8]	−16.9 [−25.7, −8.1]
Idiopathic	Hierarchical strategy	78239.2	2551	51	44	1534.1 [1439.2, 1629.0]		50.0 [47.4, 52.6]	
	ES strategy	58517.2	2256	51	51	1147.4 [1137.8, 1157.0]	386.7 [284.7, 488.7]	44.2 [42.5, 45.9]	5.8 [2.2, 9.4]

Cost difference = Hierarchical strategy cost−ES strategy cost, Diagnostic time-difference = Hierarchical strategy time−ES strategy time; Clinical subgroups with $n > 10$ patients were included for cost-effectiveness analysis.

TABLE 5 Alleles enriched in Chinese population

Gene	Position	Reference	Alteration	gnomAD-AC	gnomAD-AN	In house AC	In house AN	In house AF	P value	GnomAD AF	GnomAD East Asian AF	Reference transcript	cDNA change	protein change	Cohort AC
ATP7B	52 524 252	G	A	17	249 574	5	9966	0.000502	0.001	0.0000681161	0.000444988	NM_000053.3	c.2621C > T	p.Ala874Val	2
ATP7B	52 532 469	C	A	34	249 404	29	9966	0.00291	1.8E-24	0.000136325	0.0018912	NM_000053.3	c.2333G > T	p.Arg778Leu	4
CFTR	117 251 691	C	T	8	250882	0	9966	0	N/A	0.0000318875	0.000058055	NM_000492.3	c.3196C > T	p.Arg1066Cys	2
ATP7B	52 523 908	G	C	7	248 730	6	9964	0.000602	0.000004	0.0000281430	0.000389798	NM_000053.3	c.2755C > G	p.Arg919Gly	2
AGL	100 345 603	G	T	3	251 302	5	9960	0.000502	0.000004	0.0000119378	0.000163097	NM_000642.2	c.1735 + 1G > T	N/A	2
ATP7B	52 520 505	G	A	10	279 878	14	9966	0.001405	4.5E-15	0.0000357299	0.000461066	NM_000053.3	c.2975C > T	p.Pro992Leu	2
ATP7B	52 524 434	G	A	2	249 542	0	9966	0	N/A	0.00000801468	0.000111247	NM_000053.3	c.2549C > T	p.Thr850Ile	2
SLC25A13	95 818 684	CATA	-	84	251 422	0	9966	0	N/A	0.000334100	0.004568197	NM_014251.2	c.852_855delTATG	p.Met285ProfsTer2	2

The gnomAD exome database was used for analyses, N/A not applicable.

provided evidence using our in-house control individuals of Chinese Han population.

In conclusion, our study proved the diagnostic utility of gene panel sequencing followed by ES in a cohort of patients with paediatric liver disease. We also revealed the broad phenotypic and mutational spectrum underlying this heterogeneous group of diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

F.W., Y.L., S.Z., X.Q., M.Z., and N.W. conceived the conceptualization and methodology; F.W., Z.X., J.Y., L.C., P.W., A.L., Y.Z., X.Q., and M.Z. participated in clinical investigation. All authors contribute to the data curation work; F.W., Y.L., S.Z., Z.C., L.W., T.J.Z, Z.W., and N.W. performed the formal analysis; F.W., Y.L., and S.Z. performed data visualization and wrote the original draft of the manuscript. F.W. and M.Z. were responsible for the data validation. All authors critically revised the manuscript draft.

DATA AVAILABILITY STATEMENT

Individual participant data that underlie the results were reported after de-identification (text, tables, figures, and supplementary materials). The study protocol, statistical analysis plan, analytic code would be available, beginning three months and ending five years following article publication for researchers with a methodologically sound proposal. Proposals should be directed to gcmw2001@163.com. Data requestors will need to sign a data access agreement to gain access.

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