Combining Panel-Based Next-Generation Sequencing and Exome Sequencing for Genetic Liver Diseases

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Objectives To determine how advanced genetic analysis methods may help in clinical diagnosis.

Study design We report a combined genetic diagnosis approach for patients with clinical suspicion of genetic liver diseases in a tertiary referral center, using tools either tier 1: Sanger sequencing on *SLC2SA13*, *ATP8B1*, *ABCB11*, *ABCB4*, and *JAG1* genes, tier 2: panel-based next generation sequencing (NGS), or tier 3: whole-exome sequencing (WES) analysis.

Results In a total of 374 patients undergoing genetic analysis, 175 patients received tier 1 Sanger sequencing based on phenotypic suspicion, and pathogenic variants were identified in 38 patients (21.7%). Tier 2 included 216 patients (39 of tier 1-negative patients) who received panel-based NGS, and pathogenic variants were identified in 60 (27.8%). In tier 3, 41 patients received WES analysis, and 20 (48.8%) obtained genetic diagnosis. Pathogenic variants were detected in 6 of 19 (31.6%) who tested negative in tier 2, and a greater detection rate in 14 of 22 (63.6%) patients with deteriorating/multiorgan disease receiving one-step WES (P = .041). The overall disease spectrum is comprised of 35 genetic defects; 90% of genes belong to the functional categories of small molecule metabolism, ciliopathy, bile duct development, and membrane transport. Only 13 (37%) genetic diseases were detected in more than 2 families. A hypothetical approach using a small panel-based NGS can serve as the first tier with diagnostic yield of 27.8% (98/352).

Conclusions NGS based genetic test using a combined panel-WES approach is efficient for the diagnosis of the highly diverse genetic liver diseases. *(J Pediatr 2023;258:113408)*.

Since the discovery of a genetic cause for progressive familial intrahepatic cholestasis (PFIC), neonatal cholestasis comprises a large patient pool of genetic diseases.[1-6] The most common manifestations of cholestatic liver diseases include jaundice, hepatomegaly, clay stools, steatorrhea, rickets, pruritus, and failure to thrive. Many patients may have hepatic symptoms but without characteristic phenotypes for specific genetic liver diseases. For such patient without distinctive features, the diagnostic yield of genetic testing was approximately 12%.[7,8]

With the rapid progress of advanced genetic diagnosis methods, there have been several reports of using panel-based next-generation sequencing (NGS) and whole-exome sequencing (WES) to diagnose genetic liver diseases.[7-12] Several new genes related to hepatobiliary disease have been identified, such as *FXR* (NHR4), *MYO5B*, *DCDC2*, and *TFAM* defects.[13-16]

Advanced genetic analysis tools are not readily accessible in many hospitals, as the genetic analysis requires equipment, technique, expertise, and medical costs. Our team has designed a panel-based NGS for children with pediatric liver diseases. We found that gamma-glutamyl transferase levels of <75 or ≥300 U/L and patients possessing characteristic phenotypes, such as PFIC, Alagille syndrome, inborn errors of bile acid synthesis, Wilson disease, and polycystic diseases, were more likely to benefit from the panel-based analysis for genetic diagnosis. We proposed an NGS diagnosis classification that categorizes patients into high, moderate, or weak levels of genotype–phenotype correlations to facilitate patient management.[8]

NGS	Next-generation sequencing
NICCD	Neonatal intrahepatic cholestasis caused by citrin deficiency
PCR	Polymerase chain reaction
PFIC	Progressive familial intrahepatic cholestasis
WES	Whole-exome sequencing

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However, patients with rapid disease progression or multiple organ involvement may harbor other pathogenic variants not covered by the genetic panel. Our team also has reported that trio-based exome sequencing benefits decision-making for pediatric patients with severe illness,[17] whereas singleton WES has also been reported helpful for children with suspected monogenic conditions.[18] This study presents the results of a combined approach for genetic diagnosis in one medical center, aiming to investigate the utility of adding WES to the detection of the pathogenic cause of pediatric liver disease and to propose a clinically applicable algorithm.

Methods

A total of 374 patients with pediatric liver disease were referred for genetic analysis from 2005 to 2021 from a major pediatric liver transplantation center; this included outpatients and inpatients with liver disease onset at age <18 years. The decision for patient referral and the selection for genetic tests were made by the same pediatric gastroenterology group, with criteria adjusted in accordance with the availability of genetic testing modalities, while keeping the principles consistent. Genetic tests also were performed on stored DNA samples from 5 cases who died from early-onset hepatic failure during 2000-2003. We retrospectively collected the results of disease-causing genetic variants as well as clinical information and analyzed the results stratified by genetic testing methods. According to the availability of genetic test facilities and clinical judgment, 3 tiers of genetic diagnosis methods were used for these patients. This study was approved by the institutional review board of National Taiwan University Hospital.

During 2005-2014, the mainstay of analysis was Sanger sequencing. tier 1 included Sanger sequencing for *SLC2SA13*, *ATP8B1*, *ABCB11*, *ABCB4*, and *JAG1* genes based on clinical features: *SLC25A13* for neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD); *ATP8B1*, *ABCB11*, and *ABCB4* for phenotypic PFIC; or *JAG1* for Alagille syndrome. Full-length of coding sequencings or selected exons of reported hot spots using Sanger sequencing was tested in the aforementioned genes. After 2015, the mainstay test gradually shifted to panel-based and WES. Physicians may still choose tier 1 tests if a single genetic disorder disease is highly suspected by clinical, biochemical, or pathology features or mutation hot spots have been reported.

Tier 2 included panel-based NGS analysis. Patients with cholestasis or liver diseases of nonspecific phenotypes were first subjected to panel-based NGS. Tier 2 test was used for patients with or without disease-specific clinical features or for patients who had tested negative from tier 1 but still had persistent or progressive disease. The methods of panel-based NGS and information regarding a subset of patients have been reported.[8]

Tier 3 used WES for the diagnosis of monogenic liver disease manifestation in the index cases. Because of limited resources, tier 3 included 2 approaches: stepwise WES and one-step WES approach. The stepwise WES approach includes patients who tested negative in tier 2 (panel-based NGS) but with progressive or chronic disease; these patients were further subjected to WES. The criteria to choose one-step WES test included acute or chronic deteriorating liver disease without characteristic presentation of known genetic liver diseases, acute or chronic liver failure listing for liver transplantation, and acute or chronic liver disease with multiple-organ system involvements. The decision for stepwise or one-step WES was based on the evaluation and agreement by team of pediatric gastroenterologists/hepatologists and genetics.

Sanger Sequencing

Genomic DNA was extracted from peripheral blood with a Gentra Puregene Blood Kit Plus (Qiagen) according to the user's manual. Exons and exon-intron boundaries of *SLC25A13* (NM_001160210), *ATP8B1* (NM_005603), *ABCB11* (NM_003742), *ABCB4* (NM_000443), or *JAG1* (NM_000214) were amplified by polymerase chain reaction (PCR) with specific primers. The PCR products were cleaned up using a QIAquick PCR & Gel Cleanup Kit (Qiagen), and the sequences were determined by Sanger sequencing.

Panel-Based NGS

Two micrograms of double-stranded DNA that passed the quality control steps were sheared to ~600 bp by an M220 focused ultrasonicator (Covaris). Sheared genomic DNA was subjected to agarose gel, Nanodrop 2000 (Thermo Scientific), and Qubit 2.0 Fluorometer (Life Technologies) to confirm its purity and concentration. Fragmented DNA was tested for size distribution using an Agilent Bioanalyzer 2100 (Agilent Technologies), and a library for MiSeq was generated by a TruSeq DNA PCR-free LT sample preparation kit (Illumina) according to the manufacturer's instructions. To identify disease-causing variants, we designed a panel targeting jaundice-related genes.[8] This panel was further extended to 73 genes after 2019 (**Table I**). The target region was captured by a custom NimbleGen SeqCap EZ Choice Library (Roche). FASTQ files generated by MiSeq Report were used for further analysis.

Exome Sequencing

The capture kit for the exome region was executed using the SureSelect Human All Exon V6 Kit (Agilent) or KAPA HyperExome (Roche). Sequencing was performed using the HiSeq4000 or NovaSeq6000 (Illumina) sequencer with a 150-bp paired-end run. The average was 5 Gb of raw data with bam mean coverage >100X. Sequence reads were aligned to the human reference genome (GRCh37) using BWA followed by Genome Analysis Tool Kit (GATK V3.8; Broad Institute) best practice, and variant calling was accomplished by the Haplotypecaller.[19]

Variant Prioritization and Interpretation

Variants were annotated by wANNOVAR (http://wannovar.wglab.org/) or ANNOVAR, followed by an in-house pipeline.[8,17,20] Information from ClinVar (https://www.

Table I. Characteristics and comparisons of different genetic testing methods

Characteristics	Tier 1 (Sanger)	Tier 2 (panel)	Tier 3 (WES)
Gene number	5	73	>20 000
DOC	NA	>200×	~100-250×
BOC	Exon	59 genes with full-length; 14 genes with all exon region	Exons and exon/intron border
Advantage	High accessibility	High DOC and the ability to detect the intronic pathogenic variant	High BOC, most protein-coding genes
Disadvantage	Low BOC	Medium BOC	High BOC; computational resources needed
Turnaround time*	1-2 wk	4-8 wk	4-8 wk (within 2 wk in urgent patient condition)
Financial burden	Low	Low to medium	Medium to high
Expertise needed for data analysis and interpretation	Standard	Advanced	Multidisciplinary

BOC, breadth of coverage; *DOC*, depth of coverage; *NA*, not available; *WES*, Whole-exome sequencing.
*Based on the turnaround time of our institute for current clinical cases.

ncbi.nlm.nih.gov/clinvar/) also was added to the annotated file. Variants were first filtered with a minor allele frequency less than 1% in the public databases (gnomAD all population and Taiwan biobank) for frameshift, nonframeshift insertion/deletion, nonsense, severe missense, and splice-site variants. The pathogenicity of variants was classified based on the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines.[21-23] For variants located in splicing regions, SpliceAI was applied to evaluate the impact of alternating splicing sites.[24]

Genetic Diagnosis

For all the 3 tiers of tests, a positive genetic diagnosis was determined by 2 allelic genetic variations found for autosomal-recessive disease, and one allelic genetic variation detected for autosomal-dominant disease.[20] The patient phenotype was compatible with the genetic defects identified in agreement from the expert team of pediatric hepatologists and geneticists.

Results

Patients and Yield Rates of Genetic Diagnosis

We analyzed 374 patients who requested genetic analysis in the 3 tiers with 175, 177, and 22 patients, respectively (Figure 1). There were 175 patients who underwent tier 1 analysis (phenotype NICCD, PFIC, Alagille syndrome), and pathogenic variants of target genes *SLC25A13*, *ATP8B1*, *ABCB11*, *ABCB4*, or *JAG1* were identified in 38 of 175 (21.7%) patients. Of the 175 patients in tier 1, 26 (14.9 %) had received multiple assessment via Sanger sequencing due to negative results in the initial tests. In patients receiving multiple Sanger sequencing assays, only 15.4% (4/26) were detected with one candidate pathogenic

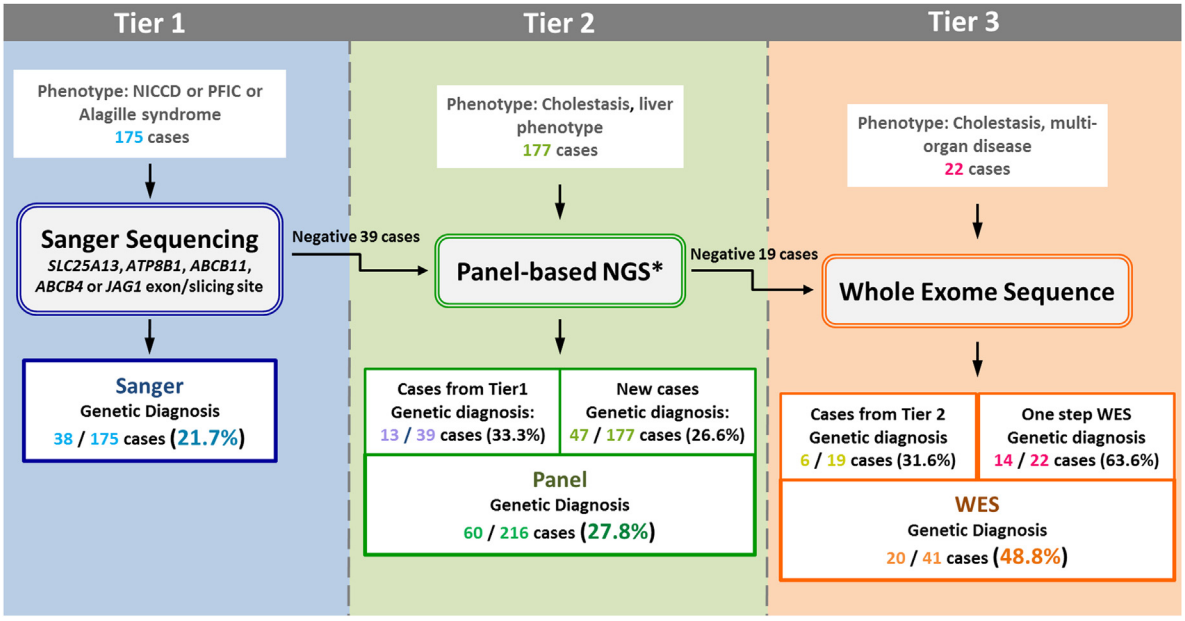

Figure 1. Summarized patient groups and diagnostic rates of tier 1, 2, 3. *Before May 2017, patients were analyzed by jaundice-associated panel version 1[8]; panel version 2 was expanded to 73 genes in patients tested after May 2017.

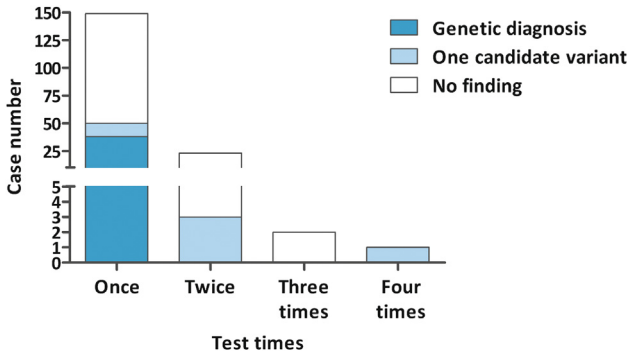

Figure 2. The test times and cases numbers with disease-causing genetic variants detected by Sanger sequencing.

variant, but none had a confirmed genetic diagnosis (Figure 2). The information of one candidate variant was provided to the clinician, but a genetic diagnosis was not made, suggesting further tier of testing when clinically indicated.

Of those patients with negative results in tier 1, 39 were subjected to panel-NGS. A total of 216 patients (39 from tier 1. and 177 new cases) in tier 2 received panel-based NGS analysis, and pathogenic variants were detected in 60 patients (60/216, 27.8%). For patients with persistent/progressive disease and negative panel-based NGS results, 19 patients were subjected to tier 3 WES analysis. We identified pathogenic variants compatible with clinical manifestation in seven (6/19, 31.6%) patients; the genes detected by WES in these patients were not included in the panel. Furthermore, 22 patients fulfilling the criteria were directly subjected to one-step WES, and 14 (14/22, 63.6%) of them were detected to have pathogenic variants (Figure 1).

The overall positive diagnostic rate of Sanger sequencing, panel-based NGS, and WES was 21.7%, 27.8%, and 48.8%, respectively. The positive rate of one-step WES was greater than that of WES from tier 2 negative cases (63.6%, vs 31.6%, $P = .041$). As an initial diagnostic tool, the detection rate of one-step WES was greater those initially tested by panel NGS (63.6% vs 26.6%, $P = .0004$). All of tier 1–positive variants could be identified by tier 2 panel-based NGS. The overall diagnostic rate of Sanger sequence and NGS panel was 27.8% (98/352).

Disease Spectrum and Distribution

Figure 3, A shows the spectrum of genes detected in Sanger sequencing, gene panel, and stepwise WES. There was high locus heterogeneity, as pathogenic variants were identified in more than 30 genes. A broader spectrum and a greater heterogeneous distribution were noted in the panel and further in WES results. In panel-based NGS, 58.3% patients harbored genes that were not covered in the tier 1 genes. In the cases diagnosed by WES, 85.0% of the patients were diagnosed with genes that were not included in the gene panel. The genetic disease spectrum and case numbers detected in each gene is shown in Figure 3, B. The most prevalent genetic defect is *SLC25A13*, as a genetic cause of NICCD in our sample. The panel identified more patients whose pathogenic variants were not covered by tier 1 Sanger sequencing, such as more *JAG1* pathogenic variants, suggesting the targeted panel is more reliable for high allelic heterogeneity genes.

Stepwise Approach and Increased Yield of Diagnosis with Advancement of Genetic Tools

For patients who were tier 1 negative, the genetic diagnostic rate was improved to 33.3% when step-up to tier 2 panel NGS test. For patients who were tier 2 negative, the genetic diagnostic was 31.6% using WES. Figure 4) shows the overall genetic spectrum and case numbers detected from the 3 tiers, showing a total of 35 genetic diseases were detected, and only 13 (37%) genetic diseases were detected in more than 2 families.

Clinical Phenotype and Patient Management

The details of patients with positive genetic findings using WES are listed in Table II, including cases from tier 2 (stepwise approach) and tier 3 (one-step approach). Of the 20 patients who had pathogenic variants detected, 10 (50.0%) were found to have previously reported genetic liver diseases in the database using phenotype search and in house analysis pipeline. Ten patients (50.0%) received trio familial analysis.[17] For 2 families with 2 siblings affected in each family, genes causing liver disease matching with the phenotype initially were not detected. Quadruple analysis using bioinformatics analysis were further applied in search of novel genetic disease, assuming an autosomal-recessive inheritance. One sibling pair, case 1 and case 2, had identical compound heterozygous variants c.2558 A > G and c.6179 C > T in a newly discovered cholestasis-associated gene, *PLEC* in our previous report.[25] Another sibling pair, case 3 and her male sibling who had died at 6 year of age, was identified to have the homozygous variant c.314 C > G on the *ZFYVE19* gene; this gene was reported to cause PFIC.[26,27]

The impact of WES on clinical management is as follows: 7 of the patients received disease-specific management, 5

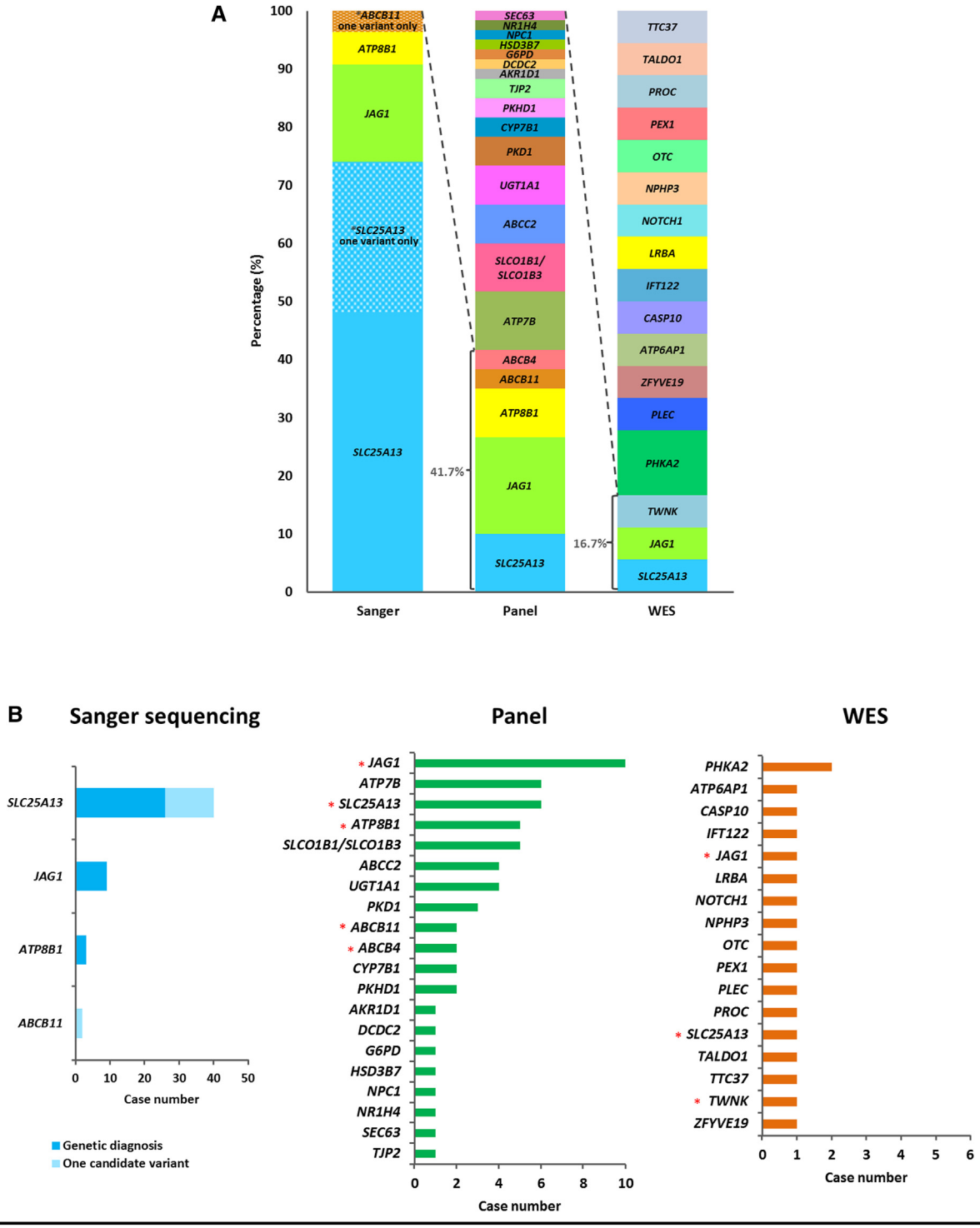

Figure 3. Genetic defects identified by different tools. **A,** The percentage of genes in the genetic-diagnosed cases detected by Sanger sequencing, panel-based NGS, or WES. The *dotted line* indicates the genes included in the lower tier. *The block with pattern indicates the cases were detected with only one pathogenic variant in *ABCB11* or *SLC25A13*. The information of one candidate variant was provided to clinician, suggesting further testing if clinically indicated, but genetic diagnosis was not made in these patients. **B,** The case number of each genetic defects diagnosed by Sanger sequencing, panel-based NGS, or WES, respectively. The *blue bars* indicate the cases with 2 variants in the autosomal-recessive disease *SLC25A13, ATP8B1, ABCB11* or one variant in the autosomal-dominant disease *JAG1*; the *light cyan bars* indicate the cases detected with only one compatible variant in autosomal-recessive disease. The *asterisk* indicates the genes included in the lower tier. The sibling cases with *PHKA2*, *PLEC*, *TJP2* defects are counted as one case from each family.

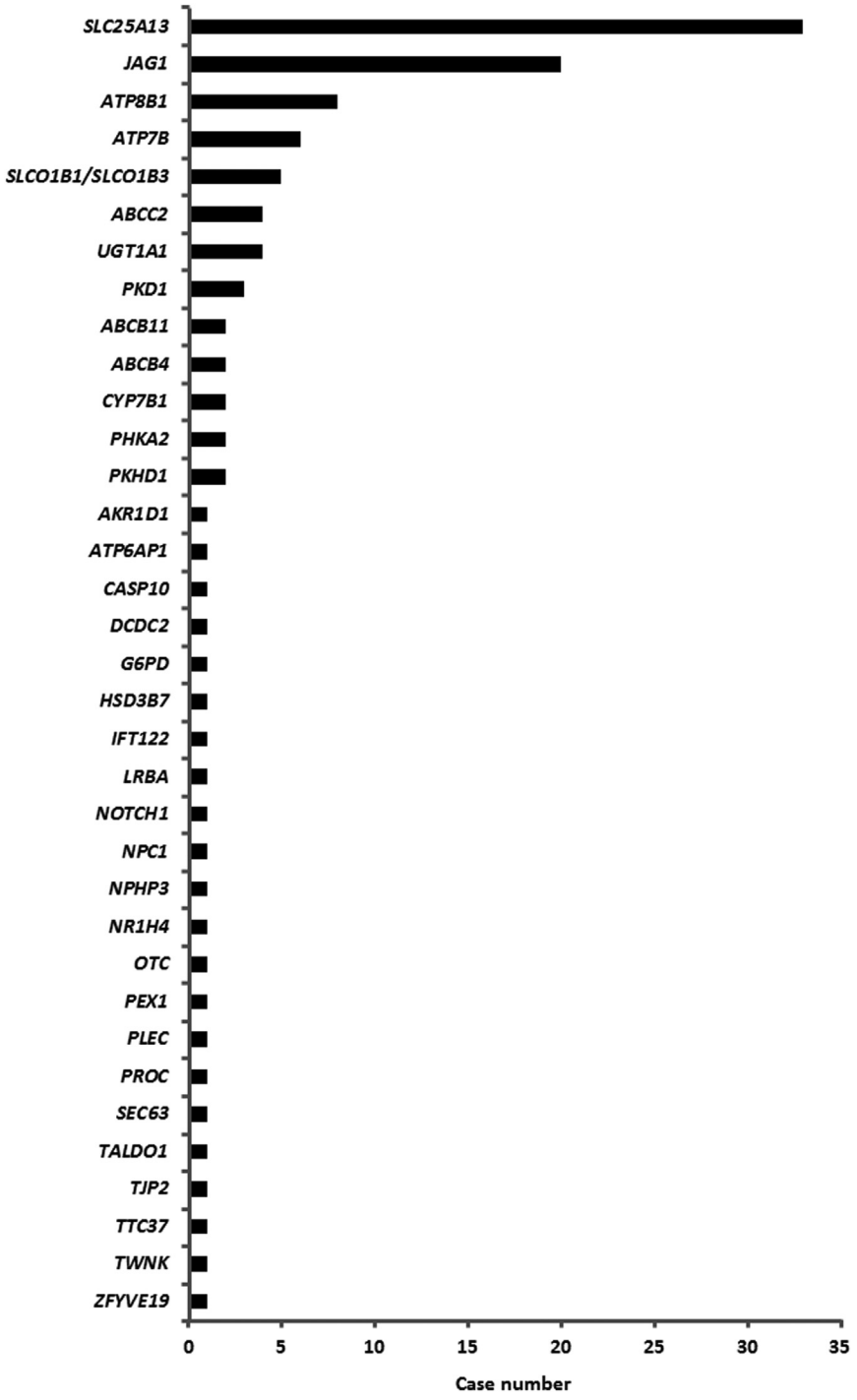

Figure 4. The sum of cases with detected genetic defects in all 3 tiers. The sibling cases of are counted as one case from each family.

received proper optimized follow-up program according to their final diagnosis, 6 initiated new subspecialist consultation/care, and 5 underwent liver transplantation. Three patients had redirection of care, including palliative care and withdrawal of live support. One patient with a *TWNK* mutation had initial presentation of neonatal cholestasis and failure to thrive. The team did not recommend liver transplantation due to the prediction of a poor prognosis. During the follow-up, the infant developed progressive neurologic deterioration. The 5-year survival of patients who had detectable genetic defects and those with negative genetic results were 84.6% vs 58.2%, respectively (P = .09) (**Figure 5**). The

Table II. Patients with genetic diagnosis identified by WES

Patients	Age/sex	Phenotype	Gene	Variant	Inheritance pattern	Genotype	Disease	Trio-WES (Y/N)	Effect on clinical management
Cases from Tier 2 (stepwise panel NGS-WES):									
1[a]	15 d/M	Familial PFIC	*PLEC*	c.2558 A > G (p.D853 G) c.6179 C > T (p.A2060 V)	AR	Compound heterozygous	New cholestasis-related gene	Y	Optimizing follow up program
2[a]	2 mo/F	Familial PFIC	*PLEC*	c.2558 A > G (p.D853 G) c.6179 C > T (p.A2060 V)	AR	Compound heterozygous	New cholestasis-related gene	Y	Liver transplantation
3[b]	birth/F	Persistent cholestasis, splenic arterial aneurysm	*ZFYVE19*	c.314 C > G(p.Ser105Ter)	AR	Homozygous	Congenital hepatic fibrosis, sclerosing cholangiopathy, and high-GGT cholestasis	Y	Optimizing follow-up program
4	3 y/M	Abnormal liver function	*PHKA2*	c.406 G > A (p.D136 N)	XLR	Hemizygous	Glycogen storage disease type IX	N	Disease-specific management
5	4 y/F	Liver cirrhosis	*IFT122*	c.452 C > T (p.S151 F) c.689 T > G (p.I230 R)	AR	Compound heterozygous	Cranioecdermal dysplasia, Sensenbrenner syndrome	Y	New subspecialist referral, optimizing follow-up program
6	2 y/M	Liver cirrhosis, epilepsy	*ATP6AP1*	c.1036 G > A (p.E346 K)	XLR	Hemizygous	Immunodeficiency 47	N	New subspecialist referral
Cases from Tier 3 (one-step WES):									
8	4 mo/F	Cholestasis Liver cirrhosis	*TALDO1*	c.500 C > T (p.T167 M) c.588 G > T (p.W196 C)	AR	Compound heterozygous	Transaldolase deficiency	Y	Liver transplantation
9	3 d/F	Hypoglycemia with cholestasis Liver cirrhosis	*SLC25A13**	c.852_855del (p.R284 fs) c.615 + 5 G > A	AR	Compound heterozygous	Citrullinemia	Y	Disease-specific management
10	Birth/M	Pachygria, corneal cloudy, cataract, abnormal liver function, cholestasis Lissencephaly, neonatal seizure	*PEX1*	c.2709_2710del (p.I903 fs) c.2391_2392del (p.S798 fs)	AR	Compound heterozygous	Zellweger syndrome	Y	Disease-specific management Redirection of care
11	Birth/M	Cholestasis, liver cirrhosis, portal hypertension, micropenis, deep-set eyes	*TTC37*	c.3426dupA (p.A1143Sfs) c.2354_2357del (p.N785Ifs)	AR	Compound heterozygous	Trichohepatoenteric syndrome	Y	Redirection of care
12	5 y/M	Chronic hepatitis, Chronic kidney disease, Hyperkalemia	*NPHP3*	c.1286 A > G (p.H429 R) c.424 C > T (p.R142X)	AR	Compound heterozygous	Renal–hepatic–pancreatic dysplasia 1	N	Optimizing follow-up program
13	3 mo/F	Failure to thrive, liver failure, thrombocytopenia, storage disease, r/o mitochondria disease	*CASP10*	c.1442 C > T (p.T481I)	AD	Heterozygous	Autoimmune lymphoproliferative syndrome, type II	N	Liver transplantation
14	10 mo/M	Portal vein thrombosis, hepatoportal sclerosis	*PROC*	c.970 G > A (p.G324S)	AD	Heterozygous	Protein C deficiency	N	Liver transplantation, optimizing follow-up program
15	5 y/F	Cholestasis,	*JAG1**	Large deletion from exon 1 to exon 5	AD	Heterozygous	Alagille syndrome	N	Liver transplantation
16	2 mo/M	Neonatal cholestasis, failure to thrive	*TWNK**	c.1523 A > G (p.Tyr508Cys) c.1844 G > C(p.Gly615Ala)	AR	Compound heterozygous	Mitochondrial DNA depletion syndrome	N	Redirection of care, new subspecialist referral
17	4 y/F	Scalp bone defect, cirrhosis, esophageal varices	*NOTCH1*	c.1451 G > T (p.Gly484Val)	AD	Heterozygous	Adams–Oliver syndrome	N	NA
18	5 y/F	Chronic hepatitis	*LRBA*	c.6914 A > G (p.Tyr2305Cys) c.1825 C > T (p.Arg609Trp)	AR	Compound heterozygous	Immunodeficiency, common variable, with autoimmunity	Y	Disease-specific management

(*continued*)

Table II. Continued

Patients	Age/sex	Phenotype	Gene	Variant	Inheritance pattern	Genotype	Disease	Trio-WES (Y/N)	Effect on clinical management
19	1 y/F	Hyperammonemia, hepatitis	*OTC*	c.663+2T > C	X-link	Heterozygous	Ornithine transcarbamylase deficiency	Y	Disease-specific management, new subspecialist referral
20[c]	2 y/M	Hepatitis	*PHKA2*	c.134 G > A (p.Arg45Gln)	XLR	Hemizygous	Glycogen storage disease, type IXa2	N	Disease-specific management, new subspecialist referral
21[c]	2 y/M	Hepatitis	*PHKA2*	c.134 G > A (p.Arg45Gln)	XLR	Hemizygous	Glycogen storage disease, type IXa2	N	Disease-specific management, new subspecialist referral

F, female; *M*, male; *N*, no; *Y*, yes.
a, b, c, indicate sibling pairs. "Redirection of care" indicates introducing palliative care or withdrawal of life support.
*The genes included in panel.

results of WES assisted in prediction of prognosis, disease-oriented preventive measures, genetic consulting, and emotional comfort of the parents.

Disease Spectrum by Functional Categories

In Figure 6, A, we present the patients diagnosed with genetic liver disease based on 4 major functional categories: disorders of metabolism disorder, membrane transport, ciliopathy and bile duct development, and others (including protein translocation, mitochondriopathies, etc). The 3 main categories comprise 90% of patients identified with genetic cause for liver diseases. In Figure 6, B, the subcategories of disease mechanisms are shown.

Discussion

An increasing number of novel genetic diseases has been added to the list of etiologies of pediatric liver disease. However, the progress in knowledge and technology has not been fully applied to clinical settings. The spectrum of disease types, timing, and indication for genetic testing is not clear. The feasibility of such testing resources is still a barrier in most primary care clinics and hospitals. The benefit of genetic diagnosis to patients has not been well recognized. In this study, we investigated the diagnostic yield and performance of 3 tiers of genetic testing, including Sanger sequencing, panel-based NGS, and WES in pediatric patients with liver disease.

Our group and others have reported a proven role of panel-based NGS in the diagnosis of inherited liver diseases, especially for pediatric cholestatic disease.[5-9] The reported positive rate ranges from 12% to 57%, owing to different study designs, referral criteria, and patient populations. WES is a powerful but expensive tool that could further identify rare pathogenic genetic variants.[12,28] Both panel and WES have been used for the diagnosis of monogenetic diseases, but their application to pediatric liver diseases is unclear. WES is mainly used on a research basis or has started to be used as clinical diagnosis tool for certain disease populations and in many settings is not covered by health care system reimbursement.

We incorporated WES into a previously adopted diagnostic investigation, designing 2 pathways. In tier 2, we applied a stepwise panel NGS-WES approach for patients with cholestasis or with a liver disease phenotype. All patients were subjected to panel NGS as an initial step. Those who had either protracted or progressive deteriorating disease but with negative NGS results received WES examination. In patients with liver diseases and multiorgan anomalies or acute or chronic deteriorating disease, we applied WES as the one-step approach. The overall positive rate in our WES cohort was approximately 50%, which is similar to previous studies. In addition, the one-step WES positive rate was higher (63.6%) than patients received stepwise panel WES-NGS (31.6%) and panel NGS-only (27.8%). This highlights the feasibility of stepwise panel WES or one-step WES approach, contributed by clinical suspicion by experienced expert team.

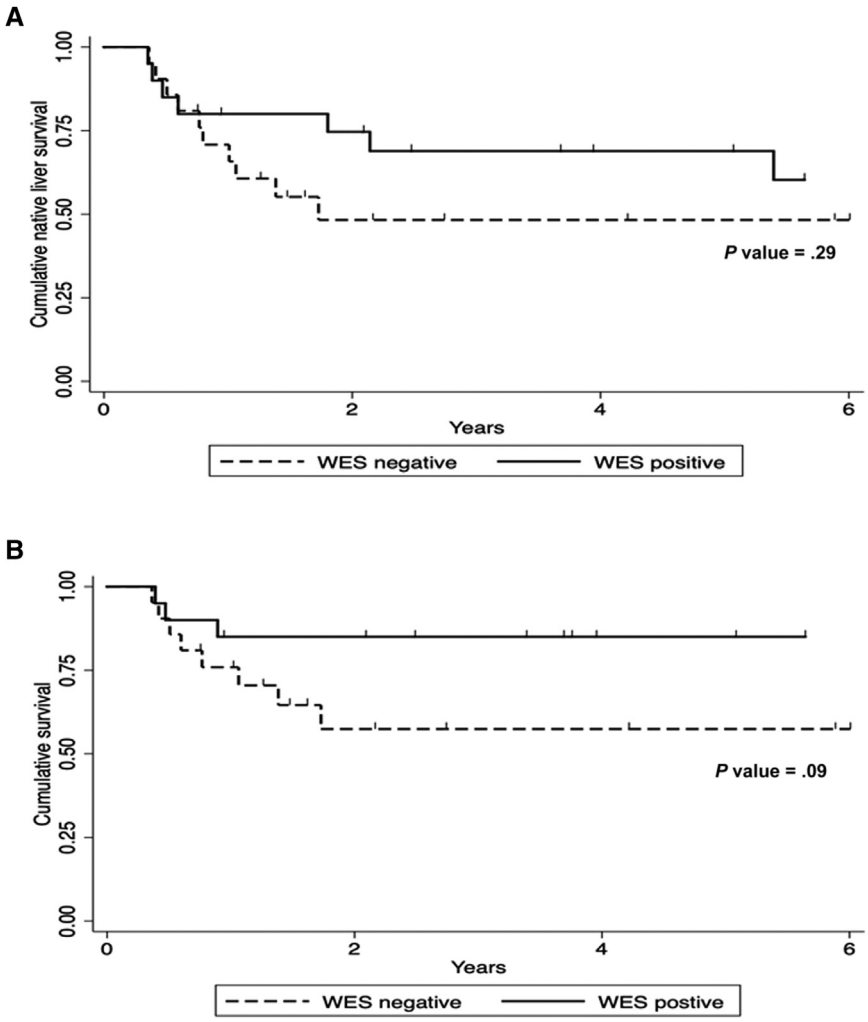

Figure 5. Kaplan–Meier survival curve for overall survival (OS) and native liver survival (NLS). Kaplan–Meier survival curve showing NLS and OS in patients with positive and negative WES diagnosis. **A,** The NLS at 5 years was 68.5% for patients with positive WES diagnosis vs 48.1% for patients with negative WES diagnosis ($P = .29$). **B,** The OS at 5 years was 84.6% for patients with positive WES diagnosis vs 58.2% for patients with negative WES diagnosis ($P = .09$).

Many of the patients in tier 2 had negative NGS results and a self-limited clinical course of cholestasis. Although the etiology of transient cholestasis in these patients was unclear in most of the cases, excessive investigation in such patients may result in unnecessary economic, executive, and psychological burdens. Many patients may be detected to have single allelic genetic variants,[8] which may be associated with susceptibility of mild disease phenotype. The interpretation of clinical significance of these results and family counseling should be cautious. deteriorating liver function, growth failure, liver fibrosis, and persistent cholestasis are reasonable criteria for further genetic analysis in these patients.

We have summarized the functional categories of genes detected in our patient population. We found the major disease categories are genes involved in small molecule metabolism, membrane transport, ciliopathy, and bile duct development. These results show the diversity of different disease mechanisms that cause similar presenting symptoms/signs.

We propose an algorithm for pediatric liver disease investigation, as shown in **Figure 7**. After evaluating the clinical manifestations and laboratory data for patients with enough clinical evidence of specific genetic liver diseases and reliable biochemical markers, we performed examinations directed to specific diseases. In patients without a clinical clue for a specific disease, panel-based NGS served as an excellent diagnostic tool. Finally, WES is the best choice for a precise diagnosis in patients with rapid deteriorating disease and patients with multiorgan involvement. WES also should be considered in patients with protracted/progressive disease and for those who fail to achieve a definite diagnosis using an NGS panel or other diagnostic tools. For institutions with readily available NGS capability and capacity, panel NGS can replace single-gene Sanger sequencing as a tier 1 test, to improve diagnostic efficacy and efficiency. The comparisons and advantages/disadvantages among these genetic studies are listed in **Table I**. We found limited

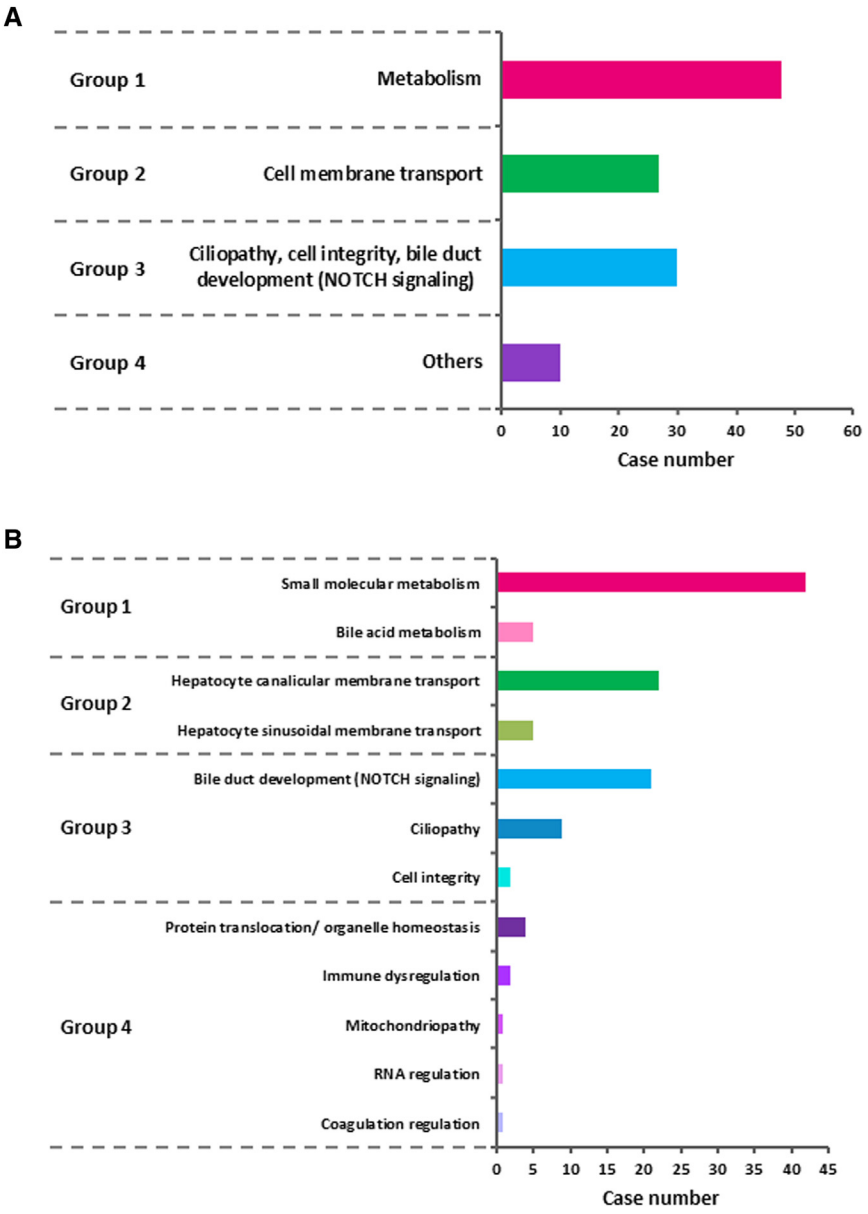

Figure 6. Functional category of genetic defects detected. Overall case numbers detected by tier 1, 2, and 3 genetic tests grouped by functional categories as **A,** major categories or **B,** subcategories.

diagnostic yield of repeatedly using Sanger sequencing in those with negative tests in the first attempt, suggesting there is little benefit to use phenotypic prediction and selective genetic testing in this regard.

The patients with nonspecific liver phenotype analyzed by the panel-based NGS as initial evaluation achieved similar diagnostic yields compared with tier 1 (26.6% vs 21.7%). However, the yield rate may be biased by the way of patient selection. In tier 1, due to limited test capacity, only patients with a highly selected phenotype were subjected to Sanger sequencing. In tier 2, due to the increased capacity of NGS, a less-stringent patient selection was used. Patients with a wider range of disease phenotype undergo the panel NGS test, and this may lower the yield rates. Sanger sequencing in patients with phenotypic diagnosis for certain diseases is still applied in some hospitals. Our results indicate that all of tier 1–positive variants can be identified by tier 2 panel-based NGS, with an estimated potential diagnostic yield of 27.8%. In our study, we demonstrate a highly diverse genetic disease spectrum underlying pediatric liver disease patient pool and justify the strategy to combine tier 1 (Sanger sequencing) and tier 2 (panel-based NGS) with a small NGS panel. As the costs decrease and NGS becomes more widely available, we suggest that a panel comprising approximately 20 genes might be sufficient as an initial tool for clinical diagnosis, followed by more-advanced and sophisticated analysis according to individual need.

The cost of WES is expected to decrease. Therefore, a combination of a small panel, WES, or WES virtual gene panel for clinical diagnosis is anticipated. The selection of panel genes

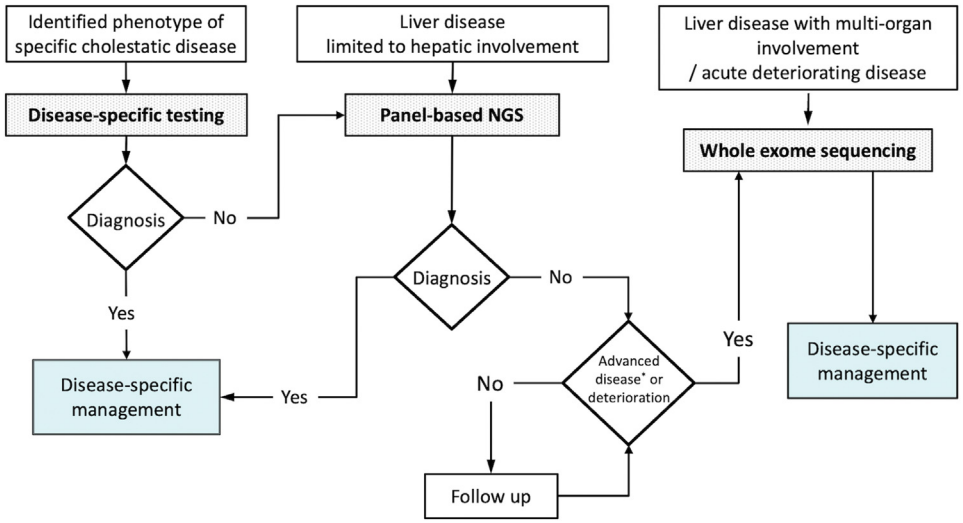

Figure 7. Proposed diagnostic algorithm of pediatric cholestatic disease in the WES era. *Advanced diseases indicate acute or chronic liver disease progression to cirrhosis of liver, or with multiple organ system anomalies.

should consider the most prevalent genetic disorders in the target population and genes that need immediate and available treatment. This may bring higher efficacy in the first step of genetic testing, serving for more patients in early stages of disease, and saving the capacity of the advanced tool for patients with priority needing WES or future whole-genome sequencing.

For those with difficult diagnoses in tier 2/3, a family approach (trio or quadruple) and whole-genome sequencing will be the next level (tier 4) of analysis. Due to a large number of genetic variations identified, a misinterpretation of causative variants may mask the true disease-causing gene/variants and result in delayed diagnosis and management.

A limitation of this study is that the patient sample was accumulated over a number of years, with different levels of genetic methodology used in each stage. Therefore, it is unfair to compare different genetic methods' usefulness for each Tier directly.

This study clearly demonstrates the expanded spectrum of genetic liver diseases as utilizing the updated genetic tools in pediatric patients with liver disease. The future era of diagnosis needs to rely on an efficient algorithm selecting patients indicated for different tools of genetic tests. The diagnosis of genetic or cholestatic liver diseases has extended beyond a physician's experience and phenotype prediction. We found that Tier 2 (stepwise panel NGS-WES approach) or Tier 3 (one-step WES approach) in this study is a preferred diagnosis algorithm for pediatric liver disease. Our combined approach is suitable for use in clinical settings to improve diagnosis and management. Further studies are required to better stratify patients for the best use of precious genetic analysis tools. ■

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Data Statement

Data sharing statement available at www.jpeds.com.

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