Panel-Based Next-Generation Sequencing for the Diagnosis of Cholestatic Genetic Liver Diseases: Clinical Utility and Challenges

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Objective To test the application of a target enrichment next-generation sequencing (NGS) jaundice panel in genetic diagnosis of pediatric liver diseases.

Study design We developed a capture-based target enrichment NGS jaundice panel containing 42 known disease-causing genes associated with jaundice or cholestasis and 10 pathway-related genes. During 2015-2017, 102 pediatric patients with various forms of cholestasis or idiopathic liver diseases were tested, including patients with initial diagnosis of cholestasis in infancy, progressive familial intrahepatic cholestasis, syndromic cholestasis, Wilson disease, and others.

Results Of the 102 patients, 137 mutations/variants in 44 different genes were identified in 84 patients. The genetic disease diagnosis rate was 33 of 102 (32.4%). A total of 79 of 102 (77.5%) of patients had at least 1 heterozygous genetic variation. Those with progressive intrahepatic cholestasis or syndromic cholestasis in infancy had a diagnostic rate of 62.5%. Disease-causing mutations, including *ATP8B1*, *ABCB11*, *ABCB4*, *ABCC2*, *TJP2*, *NR1H4* (*FXR*), *JAG1*, *AKR1D1*, *CYP7B1*, *PKHD1*, *ATP7B*, and *SLC25A13*, were identified. Nine patients had unpredicted genetic diagnosis with atypical phenotype or novel mutations in the investigational genes. We propose an NGS diagnosis classification categorizing patients into high (n = 24), moderate (n = 9), or weak (n = 25) levels of genotype–phenotype correlations to facilitate patient management.

Conclusions This panel enabled high-throughput detection of genetic variants and disease diagnosis in patients with a long list of candidate causative genes. A NGS report with diagnosis classification may aid clinicians in data interpretation and patient management. *(J Pediatr 2019;205:153-9).*

Genetic liver diseases commonly manifest as jaundice, hepatosplenomegaly, coagulopathy, or failure to thrive; the majority of patients present in infancy with overlapping symptoms. There is a complex underlying pathophysiology.[1,2] The long list of diseases that are directly or indirectly related to cholestasis includes hepatocyte transport disorders (such as progressive familial intrahepatic cholestasis [PFIC] and Dubin–Johnson syndrome), inborn errors of bile acid metabolism, bile duct development disorders (such as Alagille syndrome and polycystic liver/kidney diseases), or other metabolic disorders (such as alpha-1-antitrypsin deficiency, cystic fibrosis, and mitochondrial disorders of the liver), and diseases affecting red blood cell turnover or bilirubin conjugation.[1-5]

Previous genetic analyses of single-gene disorders based on phenotype predictions are time-consuming and labor-intensive and lead to a low yield of genetic diagnosis.[6,7] Target enrichment next-generation sequencing (NGS) has shown great potential in testing large numbers of patients with similar symptoms and signs and with multiple known candidate genes.[8-10] Target enrichment–based NGS produces only the necessary sequence information and is much more efficient at detecting disease-causing variants than whole-exome sequencing or whole-genome sequencing. However, several important issues regarding the indications for recommending patients for NGS analysis, the genes included in the NGS panel, and the diagnostic yield have not been well evaluated.

We established a targeted enrichment NGS-based genetic test platform to facilitate the diagnosis of genetic liver diseases. The aim of this study was to investigate the diagnostic roles of panel-based NGS analysis in various forms of liver diseases presenting jaundice or cholestasis. The genetic findings were further correlated with phenotypes. Based on the findings, we propose the utility of this NGS-based genetic testing approach in clinical settings, as well its application as a research tool.

ALT	Alanine transaminase
AST	Aspartate transaminase
dsDNA	Double-stranded DNA
GGT	Gamma-glutamyl transferase
NGS	Next-generation sequencing
PFIC	Progressive familial intrahepatic cholestasis
UTR	Untranslated region

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Methods

We initially included 19 pediatric patients with known genetic liver diseases that had been confirmed by Sanger sequencing as the validation group. After the validation of this group, we recruited 102 patients for the test group, including 94 patients admitted or seen at outpatient clinic at National Taiwan University Hospital, and 8 patients referred by sending samples and medical records from other hospitals during the period from 2015 to 2017. The clinical diagnosis of the enrolled patients included infant cholestasis with progressive disease; infant cholestasis with recovery; syndromic cholestasis; chronic cholestasis/idiopathic liver fibrosis/cirrhosis; Wilson disease; neonatal liver failure; liver disease associated with prematurity, total parenteral nutrition, or intestinal surgery; biliary atresia; autoimmune hepatitis/cholangitis or primary sclerosing cholangitis; and other unspecified jaundice or liver diseases. The major genetic background of patients was ethnic Han Chinese in Taiwan. Patients with cholestasis in infancy comprised the majority of the subjects and were divided into 2 groups according to follow-up outcomes: one group who achieved clinical recovery before 1 year of age and the other group with progressive disease, including patients with a clinical diagnosis of PFIC.

DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit Plus (Qiagen, Germantown, Maryland). Informed consent was obtained from the subjects or their parents. The present study was approved by the institutional review board.

Capture-Based Target Enrichment NGS Platform

A target enrichment NGS jaundice panel (NGS jaundice panel) was established. The genes tested in this panel are listed (**Table I**; available at www.jpeds.com). The genes included in this panel are known disease-causing genes that may induce jaundice or cholestasis. In addition, 10 genes known to participate in bile metabolic or regulatory pathways that have not been reported to cause human diseases also were included in this panel to explore potentially novel genes that cause human cholestasis.

Capture-based target enrichment NGS was performed using custom NimbleGen SeqCap EZ Choice Library (Roche, Pleasanton, California) DNA probes that label the target genomic sequences. In addition to the complete genomic sequence (including exons, introns, and untranslated regions [UTRs]) of all target genes, 5 kb upstream of the 5′ UTR and 3 kb downstream of the 3′ UTR genomic sequence of each gene also were included as target regions, and the total target DNA was ~4.7 Mbps. Target enrichment experiments was performed according to the NimbleGen standard protocol for Illumina library preparation.

Genomic Library Construction

Genomic DNA was analyzed with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California) to confirm its purity and concentration. Two micrograms of double-stranded DNA (dsDNA) that passed the quality control steps was sheared to ~600 bp using an M220 focused ultrasonicator (Covaris, Woburn, Massachusetts). Fragmented DNA was tested for its size distribution using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California). The MiSeq library was generated using the TruSeq DNA PCR-free LT sample preparation kit (Illumina, San Diego, California). To summarize in brief, fragmented DNA was first end-repaired to generate blunt-ended dsDNA and then size-selected using sample purification beads to retain a proper size distribution. A single deoxyadenosine was added to the size-selected blunt-ended dsDNA to facilitate the adapter ligation reaction. TruSeq-indexed adapter ligation was performed to complete the library construction. The concentration of the final library was determined by real-time quantitative polymerase chain reaction with Illumina adapter-specific primers provided by the KAPA library quantification kit (KAPA Biosystems, Wilmington, Massachusetts).

NGS with the MiSeq Platform

Libraries were denatured and sequenced on Illumina MiSeq platform with reagent v3 for paired-end sequencing (2*300 bps). Libraries were mixed with other genomic libraries and 1% Illumina PhiX control libraries to maintain the color balance for each base. Instrument control, cluster generation, image capture, and base calling were performed by Real-Time Analysis software 1.18.54 (Illumina), MiSeq Control software (MCS) 2.4.1.3 (Illumina), and MiSeq Reporter software 2.4.60.8 (Illumina). FASTQ files generated by MiSeq Report were used for alignment and variant calling and further analysis.

Variant Filtration and Sanger Sequencing for Confirmation

After annotations, variants were selected according to mutation type and allele frequency in the 1000 Genomes Project or NHLBI-ESP 6500 exome project. All frameshift and non-frameshift insertion or deletion variants, nonsense variants, and splice-site variants with allele frequencies <1% were included in subsequent analyses. The detected variants were designated as pathogenic, likely pathogenic, of uncertain significance, likely benign, or benign according to the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.[12] The genomic regions of each individual variant were amplified using the Promega GoTaq Green Master Mix with primers specific for each gene to verify the variants identified.

Results

The average proportion of reads within each library that mapped to the targeted region was 69.6 ± 0.5%, suggesting a high sensitivity of this capture reaction. The mean region coverage depth was 202.3 ± 23.7 and the mean uniformity was 92.9 ± 0.3%, indicating that the read distribution was even. Approximately 5800 single-nucleotide variants (5796 ± 226) and 600 indels (587 ± 31) were detected in every sample, regardless of the differences in read numbers, confirming the quality of this platform.

Table II. The yield rates of NGS genetic diagnosis according to disease phenotypes

Phenotypes	Total no.	Genetic diagnosis, n (%)	Heterozygous mutation, n (%)	Any mutations/variations detected, n (%)
I. Suspecting genetic diseases				
Progressive infant cholestasis	27	16 (59.2)	11 (40.7)	27 (100.0)
Syndromic cholestasis	5	4 (80.0)	0 (0.0)	4 (80.0)
Wilson disease	5	5 (100.0)	0 (0.0)	5 (100.0)
Sum	37	25 (67.6)	11 (29.7)	36 (97.3)
II. Others				
Infant cholestasis/recovery	20	4 (20.0)	12 (60.0)	16 (80.0)
Neonatal liver failure/prematurity/PNALD	14	0 (0.0)	8 (57.1)	8 (57.1)
Liver cirrhosis/chronic cholestasis	5	1 (20.0)	3 (60.0)	4 (80.0)
Biliary atresia	3	1 (33.3)	1 (33.3)	2 (66.7)
Autoimmune liver diseases	6	1 (16.7)	3 (50.0)	4 (66.7)
Other liver diseases	17	1 (5.9)	8 (47.1)	9 (52.9)
Sum	65	8 (12.3)	35 (53.8)	43 (66.2)
Total	102	33 (32.4)	42 (45.1)	79 (77.5)

PNALD, parenteral nutrition–associated liver disease.
Infant cholestasis/recovery: infant-onset cholestasis, bilirubin and transaminase levels recovered before 1 year of age.

Validation of Sequencing Results

For all variants detected in each sample, only nonsynonymous variants were selected and filtered using the following criteria: a read depth >20 and an allele frequency <1%. The genetic mutations previously identified by Sanger sequencing in 19 patients were all detected using this panel. A total of 28 variants were identified; 20 of these variants had been previously confirmed using polymerase chain reaction–based Sanger sequencing method; 8 variants were newly identified using NGS. The accuracy of NGS-based testing was 100%.

Yield Rates of Genetic Mutations Detected in the Test Group of Patients

Among the 102 patients in the test group, 137 mutations/variants in 44 different genes were identified in 84 of the tested patients. The overall yield rate of genetic diagnoses was 33 of 102 (32.4%) patients. Disease-causing mutations were identified in the *ATP8B1*, *ABCB11*, *ABCB4*, *ABCC2*, *TJP2*, *JAG1*, *AKR1D1*, *CYP7B1*, *PKD1*, *PKHD1*, *ATP7B*, *SLC25A13*, and *CFTR* genes, among others. A high proportion (77.5%, 79/102) of patients carried at least 1 heterozygous genetic mutation with predicted functional significance.

We calculated the yield rates of genetic diagnoses in patients segregated according to the phenotypes classified as described, presenting as percentage of positive genetic diagnosis among all patients in the same phenotype group (**Table II**). A greater yield of genetic diagnosis was noted in 25 of 37 (67.6%) patients with suspected genetic diseases including PFIC, Wilson disease, or syndromic cholestasis, compared with a lower yield in 8 of 65 (12.3%) patients with other liver diseases who lacked apparent features of genetic diseases, including infantile cholestasis with recovery, parenteral nutrition- or prematurity-associated cholestasis, neonatal liver failure, biliary atresia, idiopathic chronic liver disease or cirrhosis, autoimmune hepatitis/cholangitis, and primary sclerosing cholangitis.

Because cholestasis in infancy is one of the most challenging clinical conditions, we calculated the diagnostic yield of this panel among the 70 patients presenting with infant cholestasis. Of the 70 patients, those with phenotypic progressive familial intrahepatic cholestasis or syndromic cholestasis had a greater rate of genetic diagnosis compared with patients with an unspecified phenotype, including neonatal hepatitis, prematurity or parenteral nutrition/intestinal surgery–associated cholestasis, or neonatal liver failure: 62.5% vs 13.1% ($P < .0001$) (**Figure 1**, A).

We next sought to apply an earlier indication marker for NGS genetic test for cholestasis in infancy. Based on our previous report that patients with gamma-glutamyl transferase (GGT) levels at the 2 extremes (<75 U/L or >300 U/L) had worse prognosis than patients with mild to moderately elevated GGT (75-300 U/L),[13] we found patients with initial GGT levels <75 or ≥300 U/L had greater rates of genetic diagnosis (50%) than those with GGT 75-300 U/L (15%, $P = .018$) (**Figure 1**, B).

The complexity of the genetic findings also was observed in many patients carrying mutations/variations in >1 gene, shown in a heat map (**Figure 2**; available at www.jpeds.com). The additional variants in genes other than the major disease-causing gene may contribute to the pathogenesis or modification of the disease to some extent.

Different Levels of Genotype–Phenotype Correlations

Because of the complexity of genetic results generated by the expanded panel of gene lists in the NGS, clinicians not only need a NGS report listing the identified variants but also more comprehensive information that assists in data interpretation and patient management. We thus propose a classification scheme for NGS results stratified into different levels of genotype–phenotype correlations for each patient (**Table III**).

We defined a high level of correlation (level 1) as the compatibility of the genetic diagnosis with the phenotype prediction, such that biallelic *ABCB11* or *ATP8B1* mutations were detected in patients with low GGT progressive intrahepatic cholestasis. A moderate level of correlation (level 2) indicates that mutations/variations are present in known disease-causing genes in patients with an atypical phenotype, or

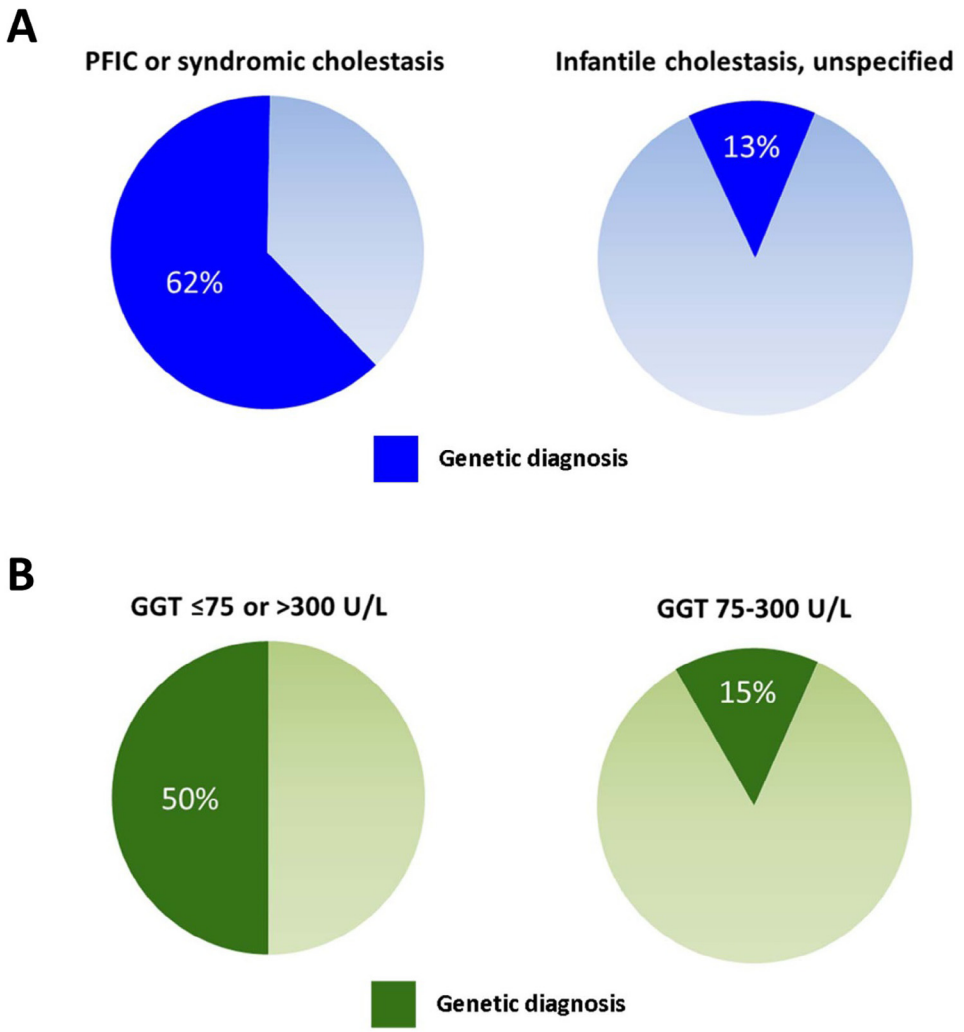

Figure 1. The rate of genetic diagnosis for infant cholestasis patients (onset <1 year) subjected to NGS jaundice panel. **A,** Based on phenotypic types, patients with phenotypic PFIC or syndromic cholestasis have a greater rate of genetic diagnosis (62.5%, 20/32) than patients without specific phenotype or are associated with prematurity, paternal nutrition/intestinal surgery (13.1%, 5/38, $P < .0001$). **B,** Based on initial GGT levels, patients with low or high GGT (<75 U/L or ≥300 U/L) have a greater rate of genetic diagnosis (50%, 21/42) than patients with mild or moderately elevated GGT levels (75-300 U/L) (15%, 3/20; $P = .018$).

mutations are present in genes involved in novel pathways related to disease pathogenesis. Level 1 and level 2 cases are listed in **Table IV** (available at www.jpeds.com).

A weak level of correlation (level 3) indicates that a heterozygous mutation was found in patients with an autosomal-recessive disease, or a missense mutation with uncertain significance was detected in patients with a dominant disorder who presented with a comparable phenotype presentation (3a) or with a noncomparable phenotype (3b). Patients without any detected mutations or who only presented with benign variants detected by this panel were classified as level 4, and either displayed (4a) or did not display (4b) a characteristic phenotype suggestive of genetic liver disease.

Disease Associated with Investigational Genes, Atypical Phenotypes, Possible Disease-Modifying Genes

We have found some patients with moderate genotype-phenotype correlations (level 2 cases) that may need further studies to verify the disease-causing role. A few examples are described to follow.

Case 25 was a female baby born at term with 3 healthy elder brothers with nonconsanguineous parents presented with neonatal cholestasis, with total and direct bilirubin level 5.6/4.6 mg/dL, aspartate transaminase (AST)/alanine transaminase (ALT) 341/308 U/L, GGT 46 U/L, bile acid 40 μM, and ammonia was 104 μg/dL. Progressive coagulopathy was noted with an international normalized ratio of up to 3.0. The

Table III. A classification scheme for different levels of genotype–phenotype correlations for each patient

Correlation levels	Description	Implications for clinicians	No.
1. **High**, definite genetic diagnosis	Pathogenic or likely pathogenic variants detected in accordance with Mendelian inheritance,* and the genetic diagnosis is compatible with patient's phenotype,† supporting disease-causing variants (mutations).	The diagnosis is made straightforward. Management of patients may follow the reported evidneces or guidelines.	24
2. **Moderate**, probable genetic diagnosis	Variants that are pathogenic, likely pathogenic, or with uncertain significance are detected* in accordance with Mendelian inheritance. Or, patients have atypical phenotype† or have variants in novel pathway related genes. Phenotype-genotype correlations are probable but need to be verified.	Management may follow the corresponding genetic disease. It should be kept in mind that because of the atypical presentation or insufficient data regarding the pathogenesis, further investigations may be warranted.	9
3. **Weak**, possible disease-causing or susceptibility mutations	3a. Phenotype compatible for defects in index gene(s), but only heterozygous variants that are pathogenic, likely pathogenic in autosomal recessive disease were found.	3a. Genetic diagnosis not made by the NGS result but the causative roles of the index gene cannot be excluded. Management may tentatively follow the guide for the index gene defects. Further investigation may be considered.‡	25
	3b. Variants with uncertain significance, likely benign, or benign, detected in patients not necessarily compatible with disease phenotype; possibly disease susceptibility gene or disease-modifying mutations.	Genetic diagnosis not made. Possibilities include that (1) the disease is not caused by single gene disorder, (2) phenotype are caused by defects in genes not included in this panel, or (3) genetic variations only contribute partly to the pathogenesis.	21
4. **Negative**	4a. Characteristic phenotype, but no variants or only benign variants are detected in the panel.	Further investigation may be considered such as whole-exome sequencing or whole-genome sequencing.	4
	4b. Phenotype unspecified, and no variants or only benign variants are detected.	Genetic diagnosis is not made. Patients may not have genetic disease or the phenotypes are caused by defects in genes not included in this panel.	19

In accordance with Mendelian inheritance: indicate biallele variants in autosomal-recessive disorders or single-allele variants in autosomal-dominant disorders.
*Variants are designated as (i) pathogenic, (ii) likely pathogenic, (iii) uncertain significance, (iv) likely benign, or (v) benign, according to recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.[12]
†Compatible phenotype: indicates that patients' phenotype is consistent with reported disease phenotype caused by the corresponding gene. Physicians are likely to choose the index gene for Sanger sequencing as priority test if NGS not available.
‡There may be possibilities that the index gene may still be the causative gene in patients with variants in only 1 allele is detected, such as a large chromosome deletion in another allele, genetic mosaicism, mutations in introns/noncoding regions affecting transcriptions, post-translational modifications affecting protein functions, or other regulatory factors involved. Further investigations in tissue specimens, cDNA, or protein assays may be considered.

alpha-fetoprotein level was >80 000 ng/mL. At 4 months of age, the bilirubin level increased to 23/16 mg/dL, AST/ALT 628/433 U/L. Abdominal sonography showed a small-sized liver with heterogeneous echotexture and splenomegaly. Liver transplantation was suggested, but the family hesitated. The patient died of sepsis at age of 5 months. The compound heterozygous mutations in the *NR1H4 (FXR)* gene, c.447_448insA (p.Asn150LysfsTer6) and c.1099C>T (p.Arg367Ter) were detected. Each parent carried 1 of the 2 heterozygous mutations. *NR1H4 (FXR)* gene was initially in our investigational gene list since 2015.

Case 26 is a male patient who had intermittent pruritus and jaundice since 3 years of age. Intermittent attacks of pruritus with jaundice once to twice per year was noted and improved with ursodeoxycholic acid and rifampin treatment. The growth and development was normal. Liver biopsy at 5 years of age revealed bland cholestasis and scattered bile plugs in dilated canaliculi, compatible with benign recurrent intrahepatic cholestasis. Sanger sequencing for *FIC1* and *BSEP* revealed no mutations in the coding sequences. At latest follow-up at age 20 years, the bilirubin level was 5.8/3.0 mg/dL, AST 66 U/L, ALT 146 U/L, alkaline phosphatase 268 U/L, GGT 53 U/L, and total serum bile acid 475 U/L. Compound heterozygous mutations in the *CFTR* gene was found in the NGS analysis.

Cases 29 and 30 presented with neonatal cholestasis with direct hyperbilirubinemia, normal or minimally elevated ALT levels, which recovered completely by 1 year of age. NGS revealed compound heterozygous *ABCC2* (MRP2) mutations in both patients.

In many of the patients, additional genetic variations were identified in genes other than the disease-causing mutations. Whether these additional genes participate in disease modification deserves further investigation.

Discussion

NGS features the high-throughput detection of mutations in multiple genes, which is suitable for genetic tests of cholestatic liver diseases because of the highly overlapping symptoms/signs and many causative genes involved in the disease pathogenesis. The latest guidelines from the North American and European Society for Pediatric Gastroenterology, Hepatology and Nutrition have recommended the use of modern, broad-based NGS sequencing in the proper clinical context.[5] However, the data remain insufficient and controversies exist on the performance and yield of the newly developed technique. Our results have shown that the NGS jaundice panel developed here efficiently achieved genetic diagnoses in clinical settings.

Conventional genetic analyses apply direct sequencing for selected genes based on the patients' phenotypes. However, the prediction of suspected genetic defects is difficult in patients with cholestatic diseases. In particular, accurate diagnostic biomarkers are not available for PFIC, and growing lists of genes have been reported to be responsible for similar phenotypes.[14-18]

However, evidence is insufficient regarding the clinical application of NGS as a high-throughput method in clinical liver diseases. In a large cohort of patients with suspected inherited cholestasis, 3 major cholestasis-related genes, *FIC1*, *BSEP*, and *MDR3*, was sequenced using the Sanger method, and 149 of 427 patients were identified to carry at least 1 disease-causing mutation.[19] High-throughput methods have been developed in the past decade in accordance with the progress of genetic analytical technology, such as resequencing chip that detects 5 genes for genetic cholestasis (*SERPINA1*, *JAG1*, *ATP8B1*, *ABCB11*, and *ABCB4*) in 2007.[20] Denaturing high-performance liquid chromatography and high-resolution melting analysis has been used to test large number of patients for detecting single-gene variants.[21,22] Recent reported NGS panels in liver diseases have incorporated smaller numbers of genes, mainly for diagnosis of PFIC.[23,24] In the present study, an expanded NGS panel was developed. The advantage is less stringent selection for phenotype required, making the panel more widely applicable.

The diagnostic yield of NGS panel largely depends on selection of patient population and threshold for calling variants. We have found that greater yield rates (67.6%) in phenotypic characteristic patients such as PFIC, Alagille syndrome, inborn errors of bile acid synthesis, Wilson disease, and polycystic diseases. However, the yield rate is still significant (12.3%) in patients without features of genetic disorders, such as infantile cholestasis with subsequent recovery (also may be categorized as neonatal hepatitis), cholestasis associated with prematurity, a history of total parenteral nutrition or surgery, autoimmune hepatitis/cholangitis, and primary sclerosing cholangitis. An earlier referral and wider application of testing is the current trend of diagnostic pathway. Our result justifies the indication of recommending patients for NGS genetic panel testing without a stringent phenotype selection at an early stage of disease. When resources are limited, a priority of selecting patients for testing may be considered, such as using initial GGT levels as a marker for cholestasis in infancy.

For the research purpose, the present NGS panel has identified new disease-causing genes by including pathway-related genes in the panel list. *NR1H4 (FXR)* initially was listed as one of the investigational genes when human diseases associated with *FXR* defect had not been reported. Subsequently, a novel infantile form of cholestatic disease with liver failure has been reported in 2 European families caused by *FXR* defect.[11]

We have found that approximately one-half of the patients with genetic diagnosis have genetic variations in more than 2 genes. Increased frequently of double or triple heterozygous gene variants have been reported to involve in intrahepatic cholestasis.[19,25-27] A greater rate of common *BSEP* and *MDR3* genetic variants was identified in patients without disease-causing mutations.[19] In addition, we unexpectedly detected heterozygous missense mutations in Jag1 or PKD1 in patients without the characteristic phenotype of Alagille syndrome or polycystic diseases. Jag1 missense mutation has been found in a small proportion (9/102 cases) of patients with biliary atresia,[28] and the association with other phenotypes is not clear. The complexity of PKD1 and PKD2 mutations in patients with polycystic kidney disease has been reported.[29] Further investigations using the database of larger numbers of NGS tested patients may help to elucidate the clinical implication of these mutations, to find the new phenotypes, and to explore the complex mechanisms of cholestatic diseases.

Given the large numbers of variants detected using NGS, interpretations of genetic diagnoses are important and closely related to decisions regarding medical interventions and family counseling. Criteria for classifying pathogenic variants have been defined and commonly applied in genetic reports.[12] A functional classification also has been proposed for disease-causing *ABCB4* variants based on biochemical studies regarding the trafficking, activity, or stability of the MDR3 protein.[30] Importantly, pathogenicity determinations of each genetic variant should be independent of the interpretations of the cause of disease in a given patient.[12] Communication between the geneticist and clinician is mandatory to define the implications of the genetic report. For the purpose of real-world applications of NGS, our proposed tool is to provide an easy-to-apply system that assists clinicians for interpretation of NGS results and determine actions for an index patient, such as disease management, family counseling, and surveillance strategies. The NGS genetic diagnosis is highly valuable in patient management for those with level 1 and level 2 genotype–phenotype correlations. For example, patients diagnosed with inborn errors of bile acid synthesis are treated with oral primary bile acid to restore liver function; patients with PFIC may be candidates for partial biliary diversion, medical treatment, or transplantation, and liver tumor surveillance.

A trend toward the rapid development of highly efficient whole-exome and whole-genome sequencing has been observed.[31,32] However, whole-exome and whole-genome sequencing produce tremendous amounts of data requiring bioinformatics analysis, cost more than the targeted enrichment NGS method, and not readily available for clinical application in many institutions in the world. We propose that target enrichment NGS panel can serve as the first-line clinical diagnostic tools. For patients for whom mutations are not detected using the NGS panel but are highly likely to have genetic disorders, whole-exome or genome sequences represent second-line analytical tools.

The present study has some limitations. Large deletions and intronic deletions/insertions may be missed by this method. Second, we screened out the reported polymorphisms with frequency greater than the set level, which are not absolutely insignificant. Third, disorders caused by epigenetic modifications cannot be detected using this method. Complex disorders in which genetic mutations/polymorphisms only participate in disease susceptibility may not be detected or explained by the results.

In summary, the high-throughput capability of the NGS-based diagnostic platform is useful in diagnosing patients with genetic liver diseases, especially infant cholestasis in clinical settings. The expanded panel of genes facilitates a wider application for this method. For complicated NGS results, a guide in the clinical interpretation for individualized patients is proposed. Further applications and investigations of this panel will

be of great benefit for improving medical decision-making processes, personalized medicine, and investigations of new disease phenotype and mechanisms. ■

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References

1. Santos JL, Choquette M, Bezerra JA. Cholestatic liver disease in children. Curr Gastroenterol Rep 2010;12:30-9.
2. Karlsen TH, Lammert F, Thompson RJ. Genetics of liver disease: from pathophysiology to clinical practice. J Hepatol 2015;62:S6-14.
3. Sundaram SS, Bove KE, Lovell MA, Sokol RJ. Mechanisms of disease: inborn errors of bile acid synthesis. Nat Clin Pract Gastroenterol Hepatol 2008;5:456-68.
4. Grochowski CM, Loomes KM, Spinner NB. Jagged1 (JAG1): structure, expression, and disease associations. Gene 2016;576:381-4.
5. Fawaz R, Baumann U, Ekong U, Fischler B, Hadzic N, Mack CL, et al. Guideline for the evaluation of cholestatic jaundice in infants: joint recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr 2017;64:154-68.
6. Chen HL, Chang PS, Hsu HC, Lee JH, Ni YH, Hsu HY, et al. Progressive familial intrahepatic cholestasis with high gamma-glutamyltranspeptidase levels in Taiwanese infants: role of MDR3 gene defect? Pediatr Res 2001;50:50-5.
7. Chen HL, Chang PS, Hsu HC, Ni YH, Hsu HY, Lee JH, et al. FIC1 and BSEP defects in Taiwanese patients with chronic intrahepatic cholestasis with low gamma-glutamyltranspeptidase levels. J Pediatr 2002;140:119-24.
8. Mamanova L, Coffey AJ, Scott CE, Kozarewa I, Turner EH, Kumar A, et al. Target-enrichment strategies for next-generation sequencing. Nat Methods 2010;7:111-8.
9. el Bahassi M, Stambrook PJ. Next-generation sequencing technologies: breaking the sound barrier of human genetics. Mutagenesis 2014;29:303-10.
10. Leproust E. Target enrichment strategies for next generation sequencing. MLO Med Lab Obs 2012;44:26-7.
11. Gomez-Ospina N, Potter CJ, Xiao R, Manickam K, Kim MS, Kim KH, et al. Mutations in the nuclear bile acid receptor FXR cause progressive familial intrahepatic cholestasis. Nat Commun 2016;7:10713.
12. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
13. Lu FT, Wu JF, Hsu HY, Ni YH, Chang MH, Chao CI, et al. gamma-Glutamyl transpeptidase level as a screening marker among diverse etiologies of infantile intrahepatic cholestasis. J Pediatr Gastroenterol Nutr 2014;59:695-701.
14. Gonzales E, Taylor SA, Davit-Spraul A, Thebaut A, Thomassin N, Guettier C, et al. MYO5B mutations cause cholestasis with normal serum gamma-glutamyl transferase activity in children without microvillous inclusion disease. Hepatology 2017;65:164-73.
15. Qiu YL, Gong JY, Feng JY, Wang RX, Han J, Liu T, et al. Defects in myosin VB are associated with a spectrum of previously undiagnosed low gamma-glutamyltransferase cholestasis. Hepatology 2017;65:1655-69.
16. Schueler M, Braun DA, Chandrasekar G, Gee HY, Klasson TD, Halbritter J, et al. DCDC2 mutations cause a renal-hepatic ciliopathy by disrupting Wnt signaling. Am J Hum Genet 2015;96:81-92.
17. Grammatikopoulos T, Sambrotta M, Strautnieks S, Foskett P, Knisely AS, Wagner B, et al. Mutations in DCDC2 (doublecortin domain containing protein 2) in neonatal sclerosing cholangitis. J Hepatol 2016;65:1179-87.
18. Stiles AR, Simon MT, Stover A, Eftekharian S, Khanlou N, Wang HL, et al. Mutations in TFAM, encoding mitochondrial transcription factor A, cause neonatal liver failure associated with mtDNA depletion. Mol Genet Metab 2016;119:91-9.
19. Droge C, Bonus M, Baumann U, Klindt C, Lainka E, Kathemann S, et al. Sequencing of FIC1, BSEP and MDR3 in a large cohort of patients with cholestasis revealed a high number of different genetic variants. J Hepatol 2017;67:1253-64.
20. Liu C, Aronow BJ, Jegga AG, Wang N, Miethke A, Mourya R, et al. Novel resequencing chip customized to diagnose mutations in patients with inherited syndromes of intrahepatic cholestasis. Gastroenterology 2007;132:119-26.
21. Chen HL, Liu YJ, Su YN, Wang NY, Wu SH, Ni YH, et al. Diagnosis of BSEP/ABCB11 mutations in Asian patients with cholestasis using denaturing high performance liquid chromatography. J Pediatr 2008;153:825-32.
22. Chen ST, Su YN, Ni YH, Hwu WL, Lee NC, Chien YH, et al. Diagnosis of neonatal intrahepatic cholestasis caused by citrin deficiency using high-resolution melting analysis and a clinical scoring system. J Pediatr 2012;161:626-31, e2.
23. Togawa T, Sugiura T, Ito K, Endo T, Aoyama K, Ohashi K, et al. Molecular genetic dissection and neonatal/infantile intrahepatic cholestasis using targeted next-generation sequencing. J Pediatr 2016;171:171-7, e1-4.
24. Vitale G, Pirillo M, Mantovani V, Marasco E, Aquilano A, Gamal N, et al. Bile salt export pump deficiency disease: two novel, late onset, ABCB11 mutations identified by next generation sequencing. Ann Hepatol 2016;15:795-800.
25. Gordo-Gilart R, Hierro L, Andueza S, Munoz-Bartolo G, Lopez C, Diaz C, et al. Heterozygous ABCB4 mutations in children with cholestatic liver disease. Liver Int 2016;36:258-67.
26. Goldschmidt ML, Mourya R, Connor J, Dexheimer P, Karns R, Miethke A, et al. Increased frequency of double and triple heterozygous gene variants in children with intrahepatic cholestasis. Hepatol Res 2016;46:306-11.
27. Dixon PH, Sambrotta M, Chambers J, Taylor-Harris P, Syngelaki A, Nicolaides K, et al. An expanded role for heterozygous mutations of ABCB4, ABCB11, ATP8B1, ABCC2 and TJP2 in intrahepatic cholestasis of pregnancy. Sci Rep 2017;7:11823.
28. Kohsaka T, Yuan ZR, Guo SX, Tagawa M, Nakamura A, Nakano M, et al. The significance of human jagged 1 mutations detected in severe cases of extrahepatic biliary atresia. Hepatology 2002;36:904-12.
29. Cornec-Le Gall E, Audrezet MP, Le Meur Y, Chen JM, Ferec C. Genetics and pathogenesis of autosomal dominant polycystic kidney disease: 20 years on. Hum Mutat 2014;35:1393-406.
30. Delaunay JL, Durand-Schneider AM, Dossier C, Falguieres T, Gautherot J, Davit-Spraul A, et al. A functional classification of ABCB4 variations causing progressive familial intrahepatic cholestasis type 3. Hepatology 2016;63:1620-31.
31. Lelieveld SH, Veltman JA, Gilissen C. Novel bioinformatic developments for exome sequencing. Hum Genet 2016;135:603-14.
32. Meng L, Pammi M, Saronwala A, Magoulas P, Ghazi AR, Vetrini F, et al. Use of exome sequencing for infants in intensive care units: ascertainment of severe single-gene disorders and effect on medical management. JAMA Pediatr 2017;171:e173438.

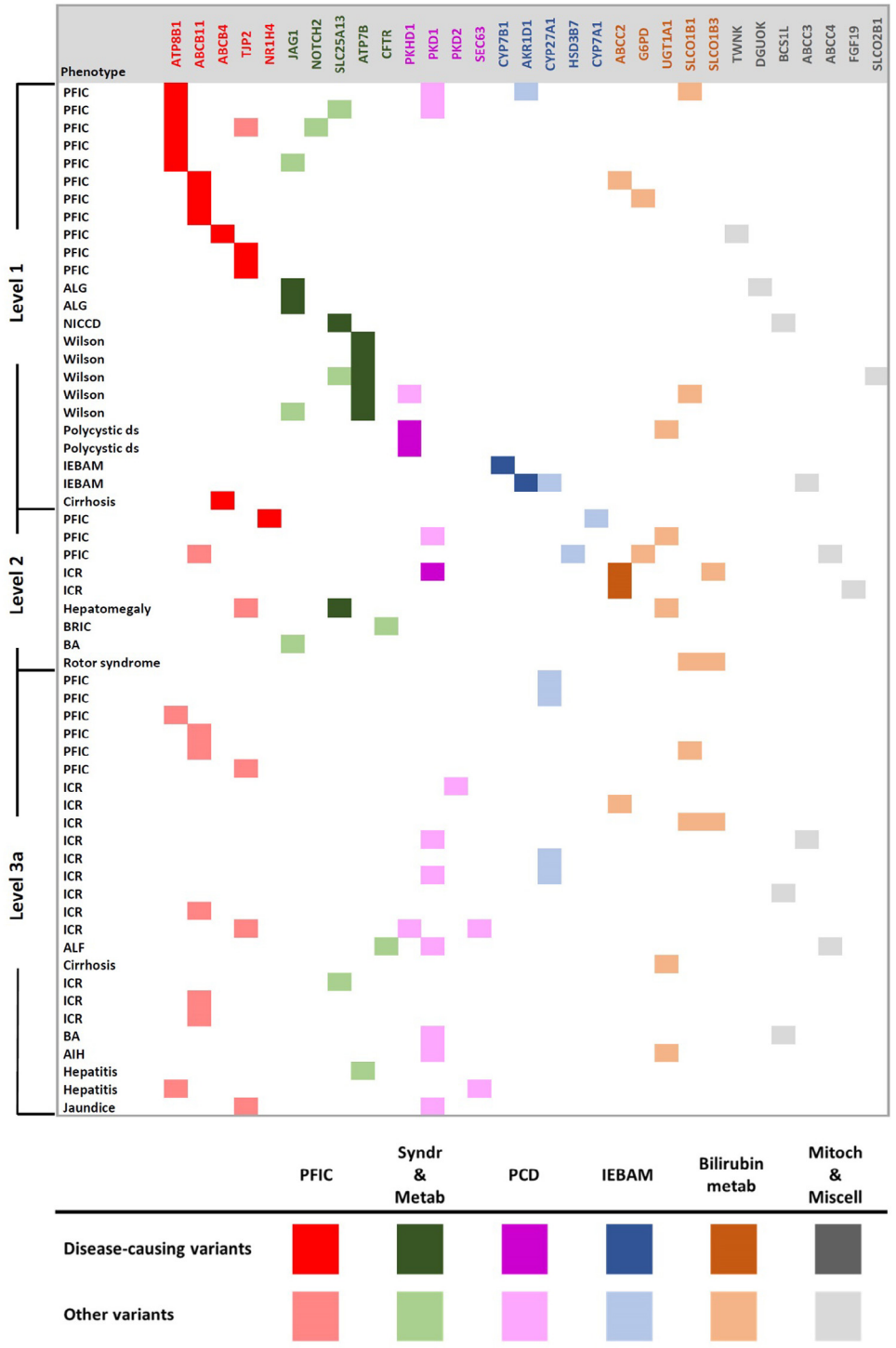

Figure 2. Representative heat map showing the genetic results obtained from NGS and the correlation with the phenotype according to levels defined in **Table III**. Patients' genetic variations detected in 31 genes of the 52 genes panel are demonstrated. In 53.4% (31/58) of patients with level 1, 2, and 3a classification, genetic variations in 2 or more genes are found. *AIH*, autoimmune hepatitis; *ALF*, acute liver failure; *ALG*, Alagille syndrome; *BA*, biliary atresia; *Bilirubin metab*, bilirubin metabolisms; *BRIC*, benign recurrent intrahepatic cholestasis; *ds*, disease; *ICR*, infant cholestasis with recovery; *IEBAM*, inborn errors of bile acid metabolisms; *Metab*, metabolic liver disease; *Miscell*, miscellaneous disorders; *Mitoch*, Mitochondrial disorder; *NICCD*, neonatal intrahepatic cholestasis caused by citrin deficiency; *PCD*, polycystic diseases; *Syndr*, syndromic cholestasis.

Table I. Gene list for NGS jaundice panel

Diseases	Gene (alias)
PFIC	ATP8B1 (FIC1), ABCB11 (BSEP), ABCB4 (MDR3), TJP2 (ZO2), NR1H4 (FXR)*
Syndromic cholestasis	JAG1, NOTCH2, VPS33B
Metabolic liver disease	SERPINA1, CFTR, SLC25A13 (CITRIN), ATP7B
Bile acid metabolic defects	CYP27A1, HSD3B7, AKR1D1, CYP7B1, BAAT, EPHX1, CYP7A1, AMACR
Mitochondrial disorder	TWNK (C10orf2), DGUOK, MPV17, POLG, BCS1L, RRM2B, SCO1, SUCLG1
Neonatal sclerosing cholangitis	CLDN1
Polycystic diseases	PKD1, PKD2, PRKCSH, SEC63, PKHD1
Bilirubin metabolism	ABCC2 (MRP2), G6PD, UGT1A1, SLCO1B1 (OATP1B1), SLCO1B3 (OATP1B3), HBA1, HBA2, HBB
Bile metabolism related genes not reported to cause human disease before	ABCB1 (MDR1), ABCC3 (MRP3), ABCC4 (MRP4), FGF19, NR1H4 (FXR)*, SLC10A1 (NTCP), SLC51A (OSTalpha, OSTA), SLC51B (OSTbeta, OSTB), SLCO1A2 (OATP1A2), SLCO2B1 (OATP2B1)

*NR1H4 (FXR) has been included in this panel at the time of development in 2014. The first report of NR1H4 to be associated with human disease was in 2016.[11]

Table IV. Tested results of the NGS panel in patients with positive genetic diagnosis (level 1 and 2 genotype–phenotype correlation)

Patient nos.	Phenotype	Genes	Genotype	HGVSc HGVSp	dbSNP	Variant pathogenicity*
Level 1						
01	Alagille syndrome	*JAG1*	het	NM_000214.2:c.2824C>T NP_000205.1:p.Gln942Ter	Novel	i
02	Alagille syndrome	*JAG1*	het	NM_000214.2:c.53_73delTCGCCCTGCTCTGTGCCCTGC NP_000205.1:p.Leu18_Leu25del	Novel	ii
		DGUOK†	het	NM_080916.2:c.679G>A NP_550438.1:p.Glu227Lys	rs104893632	ii
03	IEBAM	*CYP7B1*	hom	NM_004820.3:c.334C>T NP_004811.1:p.Arg112Ter	rs200737038	i
		ABCB1†	het	NM_000927.4:c.2677T>A NP_000918.2:p.Ser893Thr	rs2032582	v
04	IEBAM	*AKR1D1*	hom	NM_005989.3:c.853C>T NP_005980.1:p.Gln285Ter	Novel	i
		ABCC3†	het	NM_003786.3:c.4282G>A NP_003777.2:p.Val1428Met	rs532140025	iii
		CYP27A1†	het	NM_000784.3:c.491G>C NP_000775.1:p.Arg164Pro	rs148417330	iii
05	PFIC	*ATP8B1*	het	NM_005603.4:c.1798C>T NP_005594.1:p.Arg600Trp	rs780186596	i
			het	NM_005603.4:c.940G>C NP_005594.1:p.Gly314Arg	Novel	ii
		PKD1†	het	NM_001009944.2:c.10822-8delC	rs373684171	iii
		SLC25A13†	het	NM_001160210.1:c.2T>C NP_001153682.1:p.Met1?	rs541276426	iii
06	PFIC	*ATP8B1*	het	NM_005603.4:c.2081T>A NP_005594.1:p.Ile694Asn	rs541474497	ii
			het	NM_005603.4:c.279G>A NM_005603.4:c.279G>A(p.=)‡	rs761575295	i
		NOTCH2†	het	NM_024408.3:c.6104A>G NP_077719.2:p.Asn2035Ser	rs756949183	iii
		TJP2†	het	NM_001170416.1:c.2174G>A NP_001163887.1:p.Gly725Glu	rs201366118	iii
07	PFIC	*ATP8B1*	het	NM_005603.4:c.1798C>T NP_005594.1:p.Arg600Trp	rs780186596	i
			het	NM_005603.4:c.1367C>G NP_005594.1:p.Thr456Arg	rs121909104	ii
		AKR1D1†	het	NM_005989.3:c.919C>T NP_005980.1:p.Arg307Cys	Novel	iii
		PKD1†	het	NM_001009944.2:c.2039A>T NP_001009944.2:p.Tyr680Phe	rs370141157	iii
		SLCO1B1†	het	NM_006446.4:c.594delT NP_006437.3:p.Phe199SerfsTer11	rs753740379	ii
08	PFIC	*ATP8B1*	het	NM_005603.4:c.3554T>A NP_005594.1:p.Leu1185Ter	Novel	i
			het	NM_005603.4:c.2707+4A>G	Novel	ii
		JAG1†	het	NM_000214.2:c.1655C>T NP_000205.1:p.Pro552Leu	rs201785359	iii

(*continued*)

Table IV. Continued

Patient nos.	Phenotype	Genes	Genotype	HGVSc HGVSp	dbSNP	Variant pathogenicity*
09	PFIC	*ATP8B1*	het	NM_005603.4:c.2821C>T NP_005594.1:p.Arg941Ter	rs374340059	i
			het	NM_005603.4:c.2081T>A NP_005594.1:p.Ile694Asn	rs541474497	i
10	PFIC	*ABCB11*	hom	NM_003742.2:c.909-1G>A	Novel	ii
		G6PD†	het	NM_000402.3:c.577G>A NP_000393.4:p.Gly193Ser	rs137852314	iii
11	PFIC	*ABCB11*	het	NM_003742.2:c.3391G>T NP_003733.2:p.Asp1131Tyr	Novel	ii
			het	NM_003742.2:c.154C>T NP_003733.2:p.Arg52Trp	rs763526610	ii
12	PFIC	*ABCB11*	het	NM_003742.2:c.3429C>G NP_003733.2:p.Asp1143Glu	rs531704947	ii
			het	NM_003742.2:c.1772A>G NP_003733.2:p.Asn591Ser	rs11568367	iii
		ABCC2†	het	NM_000392.3:c.4465_4473GGCCCACAG NP_000383.1:p.IleThrIle1489_1491 GlyProGln	Novel	iii
13	PFIC	*TJP2*	het	NM_001170416.1:c.343A>G NP_001163887.1:p.Arg115Gly	Novel	ii
			het	NM_001170416.1:c.1377T>G NP_001163887.1:p.Tyr459Ter	Novel	i
14	PFIC	*TJP2*	het	NM_001170416.1:c.343A>G NP_001163887.1:p.Arg115Gly	Novel	ii
			het	NM_001170416.1:c.1377T>G NP_001163887.1:p.Tyr459Ter	Novel	i
15	Liver cirrhosis	*ABCB4*	hom	NM_018849.2:c.1216C>T NP_061337.1:p.Arg406Ter	rs753420538	i
		TWNK†	het	NM_021830.4:c.1495G>T NP_068602.2:p.Asp499Tyr	rs371334193	iii
16	Liver cirrhosis	*ABCB4*	het	NM_018849.2:c.3536G>T NP_061337.1:p.Gly1179Val	Novel	ii
			het	NM_018849.2:c.1744C>T NP_061337.1:p.Arg582Trp	Novel	ii
17	Polycystic liver/kidney disease	*PKHD1*	het	NM_138694.3:c.11210A>G NP_619639.3:p.Tyr3737Cys	Novel	ii
			het	NM_138694.3:c.1766_1786delGTCAGCCTCGACACCTTGTCC NP_619639.3:p.Arg589_Val596del	rs745731220	i
18	Polycystic liver/kidney disease	*PKHD1*	het	NM_138694.3:c.11314C>T NP_619639.3:p.Arg3772Ter	rs199839578	i
			het	NM_138694.3:c.6097A>G NP_619639.3:p.Arg2033Gly	rs369626030	ii
		UGT1A1†	het	NM_000463.2:c.686C>A NP_000454.1:p.Pro229Gln	rs35350960	i
19	Wilson disease	*ATP7B*	het	NM_000053.3:c.3443T>C NP_000044.2:p.Ile1148Thr	rs60431989	ii
			het	NM_000053.3:c.2393_2394delTC NP_000044.2:p.Leu798ProfsTer12	Novel	i

(*continued*)

Table IV. Continued

Patient nos.	Phenotype	Genes	Genotype	HGVSc HGVSp	dbSNP	Variant pathogenicity*
20	Wilson disease	*ATP7B*	hom	NM_000053.3:c.2975C>T NP_000044.2:p.Pro992Leu	rs201038679	i
		SLC25A13†	het	NM_001160210.1:c.1538C>T NP_001153682.1:p.Ala513Val	rs752235032	iii
		SLCO2B1†	het	NM_007256.4:c.1998C>A NP_009187.1:p.Phe666Leu	rs192050675	iii
21	Wilson disease, hepatic failure	*ATP7B*	het	NM_000053.3:c.2333G>T NP_000044.2:p.Arg778Leu	rs28942074	i
			het	NM_000053.3:c.1470C>A NP_000044.2:p.Cys490Ter	rs778675259	i
		PKHD1†	het	NM_138694.3:c.812T>A NP_619639.3:p.Leu271His	rs750887164	iii
		SLCO1B1†	het	NM_006446.4:c.1877T>A NP_006437.3:p.Leu626Ter	rs183624077	iii
22	Wilson disease	*ATP7B*	hom	NM_000053.3:c.2333G>A NP_000044.2:p.Arg778Gln	rs28942074	i
23	Wilson disease, hepatic failure	*ATP7B*	het	NM_000053.3:c.2975C>T NP_000044.2:p.Pro992Leu	rs201038679	i
			het	NM_000053.3:c.2304dupC NP_000044.2:p.Met769HisfsTer26	rs137853287	i
		JAG1†	het	NM_000214.2:c.1655C>T NP_000205.1:p.Pro552Leu	rs201785359	iii
24	NICCD	*SLC25A13*	het	NM_001160210.1:c.1402C>T NP_001153682.1:p.Arg468Ter	rs540149539	i
			het	NM_001160210.1:c.615+5G>A	rs80338717	ii
		BCS1L†	het	NM_001257344.1:c.1007+5G>A	rs373812068	iii
Level 2						
25	Infant cholestasis, liver failure	*NR1H4*	het	NM_001206993.1:c.447_448insA NP_001193922.1:p.Asn150LysfsTer6	rs879255644 (novel)	i
			het	NM_001206993.1:c.1099C>T NP_001193922.1:p.Arg367Ter	rs750222671 (novel)	i
		CYP7A1†	het	NM_000780.3:c.826G>C NP_000771.2:p.Ala276Pro	rs756548694	iii
26	Recurrent intrahepatic cholestasis	*CFTR*	het	NM_000492.3:c.1666A>G NP_000483.3:p.Ile556Val	rs75789129	v
			het	NM_000492.3:c.3289C>T NP_000483.3:p.Arg1097Cys	rs201591901	iii
27	Hepatomegaly	*SLC25A13*	het	NM_001160210.1:c.615+5G>A	rs80338717	ii
			het	NM_001160210.1:c.1402C>T NP_001153682.1:p.Arg468Ter	rs540149539	i
		UGT1A1†	het	NM_000463.2:c.686C>A NP_000454.1:p.Pro229Gln	rs35350960	i
		TJP2†	het	NM_001170416.1:c.925C>T NP_001163887.1:p.Arg309Cys	rs369572714	iii
28	Biliary atresia	*JAG1*	het	NM_000214.2:c.1655C>T NP_000205.1:p.Pro552Leu	rs201785359	iii

(*continued*)

Table IV. Continued

Patient nos.	Phenotype	Genes	Genotype	HGVSc HGVSp	dbSNP	Variant pathogenicity*
29	Infant cholestasis, recovery	*ABCC2*	het	NM_000392.3:c.1177C>T NP_000383.1:p.Arg393Trp	rs777902199	iii
			het	NM_000392.3:c.3825C>G NP_000383.1:p.Tyr1275Ter	rs554976086	i
		FGF19†	het	NM_005117.2:c.452G>A NP_005108.1:p.Arg151Gln	Novel	iii
30	Infant cholestasis, recovery	*ABCC2*	het	NM_000392.3:c.1321C>A NP_000383.1:p.Leu441Met	rs374787164	iii
			het	NM_000392.3:c.1344T>G NP_000383.1:p.Ile448Met (the c.1321C>A and c.1344 T > G are located at the same chromosome)	rs370095941	iii
			het	NM_000392.3:c.3398_3399delTT NP_000383.1:p.Tyr1134CysfsTer43	rs762914474	i
		PKD1†	het	NM_001009944.2:c.8761C>T NP_001009944.2:p.His2921Tyr	Novel	ii
		SLCO1B3†	het	NM_019844.3:c.592G>A NP_062818.1:p.Asp198Asn	rs368649517	iii
31	Infant cholestasis, cirrhosis	*PKD1*	het	NM_001009944.2:c.12455A>C NP_001009944.2:p.Lys4152Thr	rs578031762	iii
		UGT1A1†	het	NM_000463.2:c.686C>A NP_000454.1:p.Pro229Gln	rs35350960	i
			het	NM_000463.2:c.782T>C NP_000454.1:p.Val261Ala	rs772142239	iii
32	Infant cholestasis, progressive	*ABCB11*	het	NM_003742.2:c.2630_2631delGG NP_003733.2:p.Gly877AspfsTer8	Novel	i
		ABCC4	het	NM_005845.3:c.3095C>G NP_005836.2:p.Pro1032Arg	rs201038632	iii
		G6PD†	het	NM_000402.3:c.1478G>A NP_000393.4:p.Arg493His	rs72554664	iii
		HSD3B7†	het	NM_025193.3:c.521A>G NP_079469.2:p.Asn174Ser	rs750999061	iii
33	Jaundice, Rotor syndrome	*SLCO1B1*	het	NM_006446.4:c.757C>T NP_006437.3:p.Arg253Ter	rs183501729	iii
		SLCO1B3	het	NM_019844.3:c.1747+1G>A	rs373707046	iii

dbSNP, database of single nucleotide polymorphisms; *het*, heterozygous; *HGVSc*, the human genome variation society coding sequence; *HGVSp*, the human genome variation society protein sequence; *hom*, homozygous; *IEBAM*, inborn errors of bile acid metabolisms; *NICCD*, neonatal intrahepatic cholestasis caused by citrin deficiency.

Cases 1-24, level 1 genotype–phenotype correlation; cases 25-33, level 2 genotype–phenotype correlation.

*Variant pathogenicity: The detected variants were classified as (i) pathogenic, (ii) likely pathogenic, (iii) of uncertain significance, (iv) likely benign, or (v) benign according to the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.[12]

†Additional mutations/polymorphism that may be disease modifier.

‡Splicing variants.