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Diagnostic algorithm for neonatal intrahepatic cholestasis integrating single-gene testing and next-generation sequencing in East Asia

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Abstract

Background and Aim: Advances in molecular genetics have uncovered causative genes responsible for neonatal cholestasis. Panel-based next-generation sequencing has been used clinically in infants with neonatal cholestasis. We aimed to evaluate the clinical application of single-gene testing and next-generation sequencing and to develop a diagnostic algorithm for neonatal intrahepatic cholestasis.
Methods: From January 2010 to July 2021, patients suspected of having neonatal intrahepatic cholestasis were tested at the Seoul National University Hospital. If there was a clinically suspected disease, single-gene testing was performed. Alternatively, if it was clinically difficult to differentiate, a neonatal cholestasis gene panel test containing 34 genes was performed.
Results: Of the total 148 patients examined, 49 (33.1%) were received a confirmed genetic diagnosis, including 14 with Alagille syndrome, 14 with neonatal intrahepatic cholestasis caused by citrin deficiency, 7 with Dubin–Johnson syndrome, 5 with arthrogryposis-renal dysfunction-cholestasis syndrome, 5 with progressive familial intrahepatic cholestasis type II, 1 with Rotor syndrome, 1 with Niemann-Pick disease type C, 1 with Kabuki syndrome, and 1 with Phenylalanyl-tRNA synthetase subunit alpha mutation. Sixteen novel pathogenic or likely pathogenic variants of neonatal cholestasis were observed in this study. Based on the clinical characteristics and laboratory findings, we developed a diagnostic algorithm for neonatal intrahepatic cholestasis by integrating single-gene testing and next-generation sequencing.
Conclusions: Alagille syndrome and neonatal intrahepatic cholestasis caused by citrin deficiency were the most common diseases associated with genetic neonatal cholestasis. Single-gene testing and next-generation sequencing are important and complementary tools for the diagnosis of genetic neonatal cholestasis.

Introduction

Neonatal cholestasis, which affects approximately 1 in 2500 patients at birth, is a disease that occurs due to disorders in the formation or circulation of bile.[1] Symptoms of neonatal cholestasis include jaundice, acholic stools, and hepatosplenomegaly. The most common etiology is biliary atresia, but genetic cholestasis and congenital metabolic disorders also account for a large proportion of cases.[2] Advances in molecular genetics have uncovered causative genes responsible for neonatal cholestasis, previously classified as an idiopathic disease.[2] Diseases caused by these genes include alagille syndrome (ALGS), neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), progressive familial intrahepatic cholestasis (PFIC), and other diseases.[3] Because of the genetic diversity and heterogeneity of neonatal cholestasis, the need for genetic testing has emerged, which led to the use of single-gene testing for diagnosis. However, in the case of low gamma glutamyl peptidase (GGT) PFIC, several causative genes have been identified.[4–7] In this situation, next-generation sequencing (NGS), including targeted gene panel, whole exome sequencing, and whole genome sequencing have enabled the massive sequencing of various related genes and are much more efficient in detecting disease-causing variants.[8,9] However, appropriate clinical use of both single-gene testing and NGS has not been established.

The aim of this study was to examine the etiology of neonatal intrahepatic cholestasis in Korea and to evaluate the clinical application of single-gene testing and NGS. In addition, a diagnostic algorithm for neonatal intrahepatic cholestasis was suggested by

comparing the clinical characteristics and laboratory findings of the patients according to the diagnosis.

Methods

Neonatal cholestasis was defined either as a serum direct bilirubin (Db) level > 1.0 mg/dL when the serum total bilirubin (Tb) level was ≤ 5.0 mg/dL or a serum Db level > 20% when the serum Tb level was > 5.0 mg/dL.[1] Patients who had cholestasis within the first 12 months of age and persisting > 2 weeks were included, while those with extrahepatic cholestasis such as biliary atresia or choledochal cyst were excluded. In addition, patients with sepsis, metabolic disorders, or endocrine abnormalities were excluded based on their medical history and laboratory findings.

Study population and data collection. Over the past 11 years (January 2010 to August 2021), 148 patients suspected of having neonatal intrahepatic cholestasis at Seoul National University Hospital were tested. The clinical characteristics and laboratory findings were retrospectively analyzed. The clinical characteristics included sex, gestational age, birth weight, age at onset, and prognosis. The laboratory findings included levels of total protein (TP), albumin, Tb, Db, aspartate transaminase (AST), alanine transferase (ALT), GGT, cholesterol, INR, ammonia, and alkaline phosphatase. Laboratory results were based on peak values.

Single-gene testing and neonatal cholestasis gene panel. *JAG1* sequencing and multiplex ligation-dependent probe amplification were performed when neonatal cholestasis was observed, and at least one of the remaining four main criteria for ALGS was met, including dysmorphic face, congenital heart disease, vertebral anomalies, and posterior embryotoxon.[10] *SLC25A13* sequencing and PCR for the IVS16ins3kb mutation were performed based on laboratory findings, including abnormalities in serum amino acid profile indicating citrullinemia and hypoproteinemia.[11] *ATP8B1/ABCB11* sequencing was performed when cholestasis was progressive and the GGT level was low with elevated liver transaminase activity.[12] *VPS33B* sequencing was performed when neonatal cholestasis was accompanied by arthrogryposis or renal dysfunction.[13] *ABCC2* sequencing was performed when AST and ALT levels were normal.[14] If clinical identification or differentiation of neonatal cholestasis was challenging, a neonatal cholestasis gene panel was conducted. A total of 34 causative genes of neonatal cholestasis were included in the neonatal cholestasis gene panel: *ABCB11*, *ABCB4*, *ABCC2*, *AKR1D1*, *AMACR*, *ATP8B1*, *BAAT*, *CLDN1*, *CYP27A1*, *CYP7A1*, *CYP7B1*, *DGUOK*, *DHCR7*, *FAH*, *HSD3B7*, *JAG1*, *MPV17*, *NOTCH2*, *NPC1*, *NPC2*, *NR1H4*, *PKHD1*, *POLG*, *PRKC SH*, *SERPINA1*, *SLC10A1*, *SLC10A2*, *SLC25A13*, *SLCO1B1*, *SLCO1B3*, *TJP2*, *TRMU*, *VIPAS39*, *VPS33B*. The panel only included exon regions, excluding introns.

The construction of pre-capture libraries (Illumina, Inc., San Diego, CA, USA) and capture process (Agilent Technologies, Santa Clara, CA, USA) was performed according to the manufacturer's protocols. The captured libraries were sequenced using MiSeqDx (Illumina, Inc., San Diego, CA, USA). Raw sequence data were analyzed using NextGENe software (SoftGenetics, State College, PA, USA) and annotated with ANNOVAR (http://annovar.openbioinformatics.org). Common variants were filtered using gnomAD (http://gnomad.broadinstitute.org) and KRG (http://coda.nih.go.kr/coda/KRGDB) databases. The Human Gene Mutation Database and Clinvar were used to search for known pathogenic variants. The sequence variant was evaluated with a computational (in silico) predictive program using PolyPhen-2, SIFT, and MutationTaster. The effect of sequence variants is determined at the nucleotide and amino acid levels, and the potential impact of the variants on the protein can be observed. The pathogenicity of sequence variants was evaluated using the 2015 ACMG guidelines.[15]

Statistical analyses. The values of the clinical and laboratory findings among patients with a molecular genetic diagnosis were statistically analyzed. SPSS (IBM Corp Released 2017, IBM SPSS Statistics for Windows, Version 25; IBM Corp, Armonk, New York) software was used to perform statistical analysis. Kruskal–Wallis nonparametric test was performed to compare the clinical characteristics and laboratory findings between the patients according to the final diagnosis. Statistical significance was set at $P < 0.05$.

Results

Etiology of neonatal cholestasis. A total of 148 patients with neonatal intrahepatic cholestasis fulfilled our criteria and were enrolled in this study and tested with a single-gene testing or neonatal cholestasis gene panel. Of the 148 patients, 49 (33.1%) were diagnosed with genetic neonatal cholestasis (Table 1); 79 underwent single-gene testing, with 34 of them being diagnosed with genetic neonatal cholestasis, 69 underwent neonatal cholestasis gene panel testing, with 14 were diagnosed with genetic neonatal cholestasis, and one patient was diagnosed through whole exome sequencing (WES). Patients diagnosed through single-gene testing received their diagnoses 6–8 weeks after consultation, while those diagnosed through neonatal cholestasis gene panel testing were identified 8–12 weeks after consultation. However, the patient diagnosed with WES received their diagnosis over

Table 1 Etiology of genetic neonatal cholestasis

Diagnosis	*n*	%
Alagille syndrome	14	28.6
NICCD	14	28.6
Dubin–Johnson syndrome	7	14.3
ARC syndrome	5	10.2
PFIC type II	5	10.2
Rotor syndrome	1	2.0
NPC	1	2.0
Kabuki syndrome	1	2.0
FARSA mutation	1	2.0
Total	49	100%

ARC, arthrogryposis-renal dysfunction-cholestasis; FARSA, phenylalanyl-tRNA synthetase subunit alpha; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; NPC, Neimann Pick disease type C; PFIC, progressive familial intrahepatic cholestasis.

6 months after consultation. Among the cases analyzed, there were 72 cases (48.6%) of idiopathic neonatal cholestasis, 15 cases (10.1%) of total parenteral nutrition (TPN) induced cholestasis, 14 cases (9.5%) of ALGS, 14 cases (9.5%) of NICCD, 7 cases (4.7%) of Dubin–Johnson syndrome (DJS), 5 cases (3.4%) of ARC syndrome, 5 cases (3.4%) of PFIC type II, 4 cases (2.7%) of cytomegalovirus (CMV) infection, 4 cases (2.7%) of gestational alloimmune liver disease, and 1 case (0.7%) each of Rotor syndrome, Niemann–Pick disease type C (NPC), Kabuki syndrome, phenylalanyl-tRNA synthetase subunit alpha (*FARSA*) mutation, hemophagocytic lymphohistiocytosis, liver failure, heart failure, and drug induced cholestasis.

Results of neonatal cholestasis gene panel. The overall yield rate of genetic diagnoses by gene panel was 20.3% (14/69) of patients (Table S1). Among the 14 patients with genetic neonatal cholestasis, *ABCC2* mutations were found in 6, *SLC25A13* mutations were found in 4, and *NOTCH2* variant, *ABCB11*, *NPC1*, *SLCO1B3/SLCO1B3* mutations were found in 1 each. For 44 of 69 (63.8%) of any mutation/variants detected, patients remained genetically indeterminate because the variants were heterozygous pathogenic/likely pathogenic or were variants of unknown significance. No variants were detected in the neonatal cholestasis gene panel in the remaining 11 patients (15.9%). Overall, 55 (79.7%) patients with unconfirmed genetic cholestasis were diagnosed with idiopathic cholestasis, TPN-induced cholestasis, gestational alloimmune liver disease, CMV infection, and drug-induced cholestasis.

The variants of genetic neonatal cholestasis. The characteristics of the variants in the 49 patients with genetic neonatal cholestasis are summarized (Table 2). For NICCD, two patients (subjects 17 and 18) were negative in the neonatal cholestasis gene panel and a 3 kb insertion of intron 16 was detected by polymerase chain reaction (PCR). The novel variants of *JAG1* were p.Gly737fsTer6, p.Asp263_Lys264delinsGlu, p.Ile233MetfsTer8, p.Asp72ThrfsTer89, p.Glu36Lys, p.Gly114AlafsTer47, p.Cys234Ter, and p.Ala26SerfsTer47. The novel variant of *SLC25A13* was p.Arg492Trp, the novel variants of *ABCB11* were p.Arg1221Lys and p.Ile1271Asn, and the novel variant of *VPS33B* was p.Trp207Ter. These 16 novel variants were not observed in the normal population but were located at highly conserved loci in various species. All variants were considered pathogenic or likely pathogenic according to the American College of Medical Genetics and Genomics (ACMG) classification.

Clinical characteristics and laboratory findings. The clinical characteristics and laboratory findings according to the final diagnosis are shown in Table 3. Ninety-seven (65.5%) patients were male and fifty-one (34.5%) were female. The median gestational age was 38 weeks (range, 23.1–42.9), and the median age of onset was 2 weeks (range, 0–44). The gestational age and birth weight of TPN-induced cholestasis were significantly lower than those of NICCD, ALGS, idiopathic, and PFIC type II. When comparing premature and non-premature patients, TPN-induced cholestasis was statistically significantly higher in the premature group. Additionally, although not statistically significant, the rate of transient cholestasis was also higher in the premature group (Table S2). The differences in laboratory findings of the patients according to the final diagnosis are shown in Figure 1. Serum TP and Alb levels in the NICCD group were significantly lower than those in the DJS, ALGS, PFIC type II, and idiopathic groups (Fig. 1a). AST/ALT ratio of NICCD-induced cholestasis was significantly higher than that of the ALGS, PFIC type II, and idiopathic groups (Fig. 1c). The prothrombin time (INR) of NICCD was significantly higher than that of DJS, ALGS, ARC syndrome, and idiopathic cholestasis (Fig. 1e). No significant differences were found in the Tb, Db, cholesterol, and ammonia levels between the groups. Cholestasis was normalized within an average of 2 months for NICCD and idiopathic cholestasis and 2.5 months for TPN-induced cholestasis.

The patient with the *NOTCH* variant (subject 01) did not meet the classic criteria for ALGS diagnosis. He showed normal facial features with no abnormalities in the heart, ocular, and skeletal system, but had hydronephrosis in the left kidney. In the NICCD group, 3 of 14 (21.4%) patients had normal neonatal screening test (NST) results at the initial evaluation before 2 months of age but were found to be abnormal at follow-up after 2 months of age.

One patient (subject 49) with neonatal cholestasis, chronic diarrhea, hyperammonemia, and hypoalbuminemia after birth was not genetically diagnosed using the neonatal cholestasis gene panel. Therefore, whole-exome sequencing was performed on the patient and the parents, and the *FARSA* variant was found to be a carrier in the patient's parents and two pairs in the patient.

Diagnostic algorithm for neonatal intrahepatic cholestasis. Based on clinical characteristics and laboratory findings, we developed a diagnostic algorithm for neonatal cholestasis (Fig. 2). First, patients suspected of having neonatal intrahepatic cholestasis are examined to rule out any abnormalities using the following evaluations: medical history, vital signs, NST, C-reactive protein level, thyroid function test, infection, and metabolic disease. Consequently, single-gene testing is considered when clinical symptoms suggest specific diseases such as DJS, NICCD, ALGS, and ARC syndrome. The protocol for single-gene testing involved *ABCC2* sequencing when AST and ALT levels are normal; *SLC25A13* sequencing when coagulopathy is accompanied by citrullinemia; *JAG1/NOTCH2* sequencing when a patient is presenting with a dysmorphic face, butterfly vertebra, and peripheral pulmonary stenosis; or *VPS33B/VIPAS39* sequencing when the serum GGT level is low and there is joint contracture or renal tubular dysfunction. If the diagnosis was not confirmed from previous single-gene testing, a neonatal cholestasis gene panel was performed. Subsequently, cases where it was difficult to differentiate a specific disease were confirmed as transient cholestasis if the cholestasis resolved within 2 months. Transient cholestasis is further classified as TPN-induced cholestasis if a history of TPN, preterm birth and very low birth weight is presented. Alternatively, whole-exome sequencing could be considered if the diagnosis is not confirmed by the neonatal cholestasis gene panel and the patient's cholestasis persists.

Table 2 Characteristics of the variants in the 49 patients with a genetic neonatal cholestasis

Subjects	Diagnostic tool	Diagnosis	Affected gene	Variant	ACMG criteria	Classification
01	Neonatal cholestasis gene panel	ALGS	*NOTCH2*	c.5104C > T, p.Arg1702Ter	PVS1, PM2, PP3	Pathogenic variant
02	*JAG1* single-gene testing	ALGS	*JAG1*	c.439C > T, p.Gln147Ter	PVS1, PM2, PP3, PP5	Pathogenic variant
03	*JAG1* single-gene testing	ALGS	*JAG1*	c.550C > T, p.Arg184Cys	PM1, PM2, PP3, PP5	Likely pathogenic variant
04	*JAG1* single-gene testing	ALGS	*JAG1*	†c.2210del, p.Gly737GlufsTer6	PVS1, PM2	Likely pathogenic variant
05	*JAG1* single-gene testing	ALGS	*JAG1*	c.53_73del, p.Leu18_Leu24del	PVS1, PM2, PP3	Pathogenic variant
06	*JAG1* single-gene testing	ALGS	*JAG1*	c.551G > A, p.Arg184His	PM1, PM2, PP3, PP5	Likely pathogenic variant
07	*JAG1* single-gene testing	ALGS	*JAG1*	†c.789_791delTAA, p.Asp263_Lys264delinsGlu	PVS1, PM2	Likely pathogenic variant
08	*JAG1* single-gene testing	ALGS	*JAG1*	c.133G > T, p.Val45Leu†c.699_700delTT, p,Ile233MetfsTer8	PM1, PM2, PP2, PP3 PVS1, PM2	Pathogenic variant Likely pathogenic variant
09	*JAG1* single-gene testing	ALGS	*JAG1*	†c.213_215delinsCA, p.Asp72ThrfsTer89	PVS1, PM2	Likely pathogenic variant
10	*JAG1* single-gene testing	ALGS	*JAG1*	†c.106G > A, p.Glu36Lys	PM1, PM2, PP2, PP3	Likely pathogenic variant
11	*JAG1* single-gene testing	ALGS	*JAG1*	†c.341delG, p.Gly114AlafsTer47	PVS1, PM2	Likely pathogenic variant:
12	*JAG1* single-gene testing	ALGS	*JAG1*	c.551G > A, p.Arg184His	PM1, PM2, PP3, PP5	Likely pathogenic variant
13	*JAG1* single-gene testing	ALGS	*JAG1*	†c.702C > A, p.Cys234Ter	PVS1, PM2, PP3	Pathogenic variant
14	*JAG1* single-gene testing	ALGS	*JAG1*	†c.74dupG, p.Ala26SerfsTer47	PVS1, PM2, PP3	Pathogenic variant
15	Neonatal cholestasis gene panel	NICCD	*SLC25A13*	c.1177 + 1G > A, c.852_855del, p.Met285ProfsTer2	PM1, PM2, PP3, PP5 PVS1, PM2, PP3	Likely pathogenic variant Pathogenic variant
16	Neonatal cholestasis gene panel	NICCD	*SLC25A13*	c.1177 + 1G > A,homozygote	PM1, PM2, PP3, PP5	Likely pathogenic variant
17	Neonatal cholestasis gene panel	NICCD	*SLC25A13*	IVS16ins3KB, homozygote	PM1, PM2, PP3, PP5	Likely pathogenic variant
18	Neonatal cholestasis gene panel	NICCD	*SLC25A13*	IVS16ins3KB, homozygote	PM1, PM2, PP3, PP5	Likely pathogenic variant
19	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.526G > A, p.Gly176Arg c.1079G > T, p.Arg360Leu	PM1, PM2, PP2, PP3 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant
20	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.674C > A, p.Ser225Ter c.1177 + 1G > A	PVS1, PM2, PP3, PP5 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
21	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.852_855delGTAT, p.Met285ProfsTer2	PVS1, PM2, PP3	Pathogenic variant
22	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.852_855delGTAT, p.Met285ProfsTer2	PVS1, PM2, PP3	Pathogenic variant
23	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.674C > A, p.Ser225Ter IVS16ins3KB: heterozygote	PVS1, PM2, PP3, PP5 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant

(Continues)

Subjects	Diagnostic tool	Diagnosis	Affected gene	Variant	ACMG criteria	Classification
24	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	†c.1474C > T, p.Arg492Trp IVS16ins3KB: heterozygote	PM1, PM2, PP2, PP3 PM1, PM2, PP3, PP5	Likely pathogenic variant Likely pathogenic variant
25	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.674C > A, p.Ser225Ter c.1177 + 1G > A	PVS1, PM2, PP3, PP5 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
26	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.674C > A p.Ser225Ter, c.1177 + 1G > A	PVS1, PM2, PP3, PP5 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
27	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.1177 + 1G > Ac.852_855del p.Met285ProfsTer390	PM1, PM2, PP3, PP5 PVS1, PM2, PP3	Likely pathogenic variant Pathogenic variant
28	*SLC25A13* single-gene testing	NICCD	*SLC25A13*	c.852_855del, p.Met285ProfsTer2 c.1177 + 1G > A	PVS1, PM2, PP3 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
29	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.2302C > T, p.Arg768Trp, homozygote	PM1, PM2, PP3, PP5	Likely pathogenic variant
30	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.298C > T, p.Arg100Ter c.2302C > T, p.Arg768Trp	PVS1, PM2, PP3 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
31	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.2302C > T, p.Arg768Trp c.2443C > T, p.Arg815Ter	PM1, PM2, PP3, PP5 PVS1, PM2, PP3, PP5	Likely pathogenic variant Pathogenic variant
32	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.2302C > T, p.Arg768Trp c.2224G > A, p.Asp742Asn	PM1, PM2, PP3, PP5 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant
33	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.298C > T, p.Arg100Ter c.2302C > T, p.Arg768Trp	PVS1, PM2, PP3 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
34	Neonatal cholestasis gene panel	DJS	*ABCC2*	c.298C > T, p.Arg100Ter c.3928C > T, p.Arg1310Ter	PVS1, PM2, PP3 PVS1, PM2, PP3	Pathogenic variant Pathogenic variant
35	*ABCC2* single-gene testing	DJS	*ABCC2*	c.2439 + 2 T > C c.351_355dupCCAAT, p.Tyr119SerfsTer34	PM1, PM2, PP3, PP5 PVS1, PM2, PP3	Likely pathogenic variant Pathogenic variant
36	Neonatal cholestasis gene panel	PFIC type II	*ABCB11*	c.3812 T > A, p.Ile1271Asn, homozygote	PM1, PM2, PP2, PP3	Likely pathogenic variant
37	*ABCB11* single-gene testing	PFIC type II	*ABCB11*	c.2281_2284delGGTG, p.Gly761GlnfsTer3 c.667C > T, p.Arg223Cys	PVS1, PM2, PP3 PM1, PM2, PP2, PP3	Pathogenic variant Likely pathogenic variant
38	*ABCB11* single-gene testing	PFIC type II	*ABCB11*	†c.2075 + 3A > G †c.3662G > A, p.Arg1221Lys	PM1, PM2, PP2, PP3 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant
39	*ABCB11* single-gene testing	PFIC type II	*ABCB11*	†c.151-9 T > G †c.3812 T > A, p.Ile1271Asn	PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant

(Continues)

Subjects	Diagnostic tool	Diagnosis	Affected gene	Variant	ACMG criteria	Classification
					PM1, PM2, PP2, PP3	
40	*ABCB11* single-gene testing	PFIC type II	*ABCB11*	c.2075 + 3A > G c.3662G > A, p.Arg1221Lys	PM1, PM2, PP2, PP3 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant
41	*VPS33B* single-gene testing	ARC	*VPS33B*	†c.621G > A, p.Trp207Ter†c.239 + 5G > A	PVS1, PM2, PP3 PM1, PM2, PP2, PP3	Pathogenic variant Likely pathogenic variant
42	*VPS33B* single-gene testing	ARC	*VPS33B*	c.403 + 2 T > A, homozygote	PM1, PM2, PP2, PP3	Likely pathogenic variant
43	*VPS33B* single-gene testing	ARC	*VPS33B*	c.403 + 2 T > A, homozygote	PM1, PM2, PP2, PP3	Likely pathogenic variant
44	*VPS33B* single-gene testing	ARC	*VPS33B*	c.403 + 2 T > A †c.239 + 5G > A	PM1, PM2, PP2, PP3 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant
45	*VPS33B* single-gene testing	ARC	*VPS33B*	c.403 + 2 T > A, homozygote	PM1, PM2, PP2, PP3	Likely pathogenic variant
46	Neonatal cholestasis gene panel	Rotor syndrome	*SLCO1B1/ SLCO1B3*	c.1738C > T, p.Arg580Ter, homozygote intronic LINE-1 insertion, homozygote	PVS1, PM2, PP3 PM1, PM2, PP3, PP5	Pathogenic variant Likely pathogenic variant
47	Neonatal cholestasis gene panel	NPC	*NPC1*	c.1145C > G, p.Ser382Ter c.2231_2233del, p.Val744del	PVS1, PM2, PP3 PVS1, PM2, PP3	Pathogenic variant Pathogenic variant
48	*KMT2D* single-gene testing	Kabuki syndrome	*KMT2D*	c.7411C > T, p.Arg2471Ter	PVS1, PM2, PP3, PP5	Pathogenic variant
49	WES	*FARSA* mutation	*FARSA*	c.1424G > A, p.Arg475Gln c.1040C > T, p.Pro347Leu	PM1, PM2, PP2, PP3 PM1, PM2, PP2, PP3	Likely pathogenic variant Likely pathogenic variant

ALGS, Alagille syndrome; ARC, arthrogryposis-renal dysfunction-cholestasis; DJS, Dubin–Johnson syndrome; FARSA, phenylalanyl-tRNA synthetase subunit alpha; fs, frameshift mutation; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; NPC, Neimann Pick disease type C; PFIC, progressive familial intrahepatic cholestasis; WES, whole exome sequencing.
†Novel variants.

Discussion

This is the first study to analyze the clinical applications of single-gene testing and NGS in patients with neonatal intrahepatic cholestasis and to develop a diagnostic algorithm for the disease in Asia. In this single-center study, we investigated 148 patients with neonatal intrahepatic cholestasis, and a molecular genetic diagnosis was made in 33.1% of them (49/148). Notably, a total of 16 novel pathogenic or likely pathogenic variants of neonatal cholestasis were observed in this study. More importantly, laboratory findings and clinical characteristics of the patients varied depending on their final diagnosis. This allowed us to develop a diagnostic algorithm for neonatal intrahepatic cholestasis.

Genetic testing for neonatal cholestasis has advanced since the 2000s because extensive biochemical and histological tests to identify the etiology of neonatal cholestasis have been ineffective.[16] With advances in molecular genetics, NGS panels are being used to differentiate neonatal cholestasis; however, the diagnostic yield of panels depends heavily on the selection of patient populations.[9] For the present study, the overall yield rate of the gene panel was 20.3%. Other studies using gene panels have reported yield rates of 12%, 22%, 26%, 32%, and 61%.[9,17–20] Interestingly, a gene panel study conducted in Saudi Arabia reported a yield rate of 61%; the Middle East is likely to have a high diagnosis rate because of the high number of consanguineous marriages.[21]

In our study, genetic neonatal cholestasis was initially pre-filtered by performing single-gene testing if there were clinically suspected diseases. In addition, considering that cholestasis was normalized in 33 of the 34 patients who were diagnosed with idiopathic cholestasis in the gene panel in our study, these patients could be diagnosed with transient cholestasis. In tertiary hospitals such as our hospital, there are many premature births and low birth weight, which are risk factors for transient cholestasis.[22–24] Therefore, the diagnostic yield of the gene panel used in our study was reported to be low. In a study in Italy, the transient cholestasis rate was reduced to some extent by including only patients with cholestasis for > 6 weeks.[16]

Table 3 Clinical characteristics and laboratory findings of the patients according to final diagnosis

Characteristics	ALGS	NICCD	DJS	ARC	PFIC type II	Idiopathic	TPN induced
Sex, male (%)	8 (57%)	9 (64%)	4 (57%)	4 (80%)	3 (60%)	45 (63%)	11 (73%)
Gestational age, week, median (range)	39.3 (32.7–40.7)	39.0 (32–40.5)	38.5 (36.3–40)	37.3 (37–39.7)	38.0 (38–39.7)	38.0 (25.1–42.9)	30.4 (23.1–41)
Birth weight, g, median (range)	2495 (1340–2910)	2685 (1680–3080)	2850 (2710–3310)	2720 (1930–2980)	3470 (2760–3900)	2780 (730–4340)	1260 (520–3210)
Age of onset, week, median (range)	1 (0–12)	6 (0–12)	1 (0–4)	0 (0–4)	1 (0–44)	2 (0–28)	3 (0–24)
Total protein (g/dL)	6.1 (5.0–6.4)	4.5 (3.8–5.5)	5.9 (5.1–6.8)	5.4 (4.9–6.0)	5.9 (5.7–7.1)	5.7 (4.0–8.6)	5.3 (3.8–6.2)
Albumin (g/dL)	4.1 (3.4–4.3)	2.9 (2.4–3.8)	4.2 (3.8–4.6)	3.3 (3.0–3.9)	4.1 (4.1–5.0)	3.9 (2.9–5.2)	3.5 (2.7–4.5)
Tb (mg/dL)	7.6 (1.8–23.1)	8.3 (2.4–13.5)	11.0 (1.7–30.0)	7.2 (5.5–9.2)	5.0 (3.3–18.3)	8.6 (2.6–36.3)	10.9 (4.3–28.1)
Db (mg/dL)	5.5 (2.9–19.5)	3.9 (1.7–8.1)	6.1 (1.4–9.4)	5.5 (4.3–7.3)	3.6 (2.5–11.0)	5.1 (1.6–24.4)	8.7 (3.4–19.3)
AST (IU/L)	171 (23–442)	125 (43–344)	43 (25–71)	49 (37–70)	415 (174–2122)	146 (16–889)	201 (51–1424)
ALT (IU/L)	120 (9–419)	47 (30–81)	19.5 (11–43)	33 (27–77)	550 (191–1291)	114 (5–714)	157 (16–541)
AST/ALT	1.33 (0.76–3.33)	2.50 (1.50–4.50)	1.90 (1.20–3.30)	1.32 (0.91–1.63)	0.91 (0.66–1.64)	1.39 (0.52–13.59)	1.69 (0.94–2.59)
GGT (IU/L)	331 (70–1138)	208 (99–340)	109 (27–209)	25 (23–44)	36 (26–59)	84 (30–976)	71 (41–260)
Cholesterol (mg/dL)	163 (117–572)	145 (110–187)	153 (123–198)	203 (100–232)	202 (167–357)	154.5 (58–352)	142 (95–257)
INR	0.96 (0.81–1.10)	1.38 (1.23–3.28)	0.99 (0.91–1.10)	0.9 (0.83–1.03)	1.48 (1.02–3.27)	1.04 (0.82–2.47)	1.33 (0.94–2.6)
Ammonia (μg/dL)	76 (50–111)	93.5 (23–216)	82.5 (56–135)	81 (49–113)	100 (64–114)	91 (36–259)	116 (73–187
Duration of cholestasis (month)		2.0 (1.0–3.3)				2.2 (0.3–7.0)	2.5 (1.0–4.0)

†Kruskal–Wallis test.

DJS, Dubin–Johnson syndrome; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; ALGS, Alagille syndrome; PFIC, progressive familial intrahepatic cholestasis; ARC, arthrogryposis-renal dysfunction-cholestasis; TPN, total parenteral nutrition; Tb, total bilirubin; Db, direct bilirubin; AST, aspartate transaminase; ALT, alanine transferase; GGT, gamma glutamyl peptidase; INR, international normalized ratio.

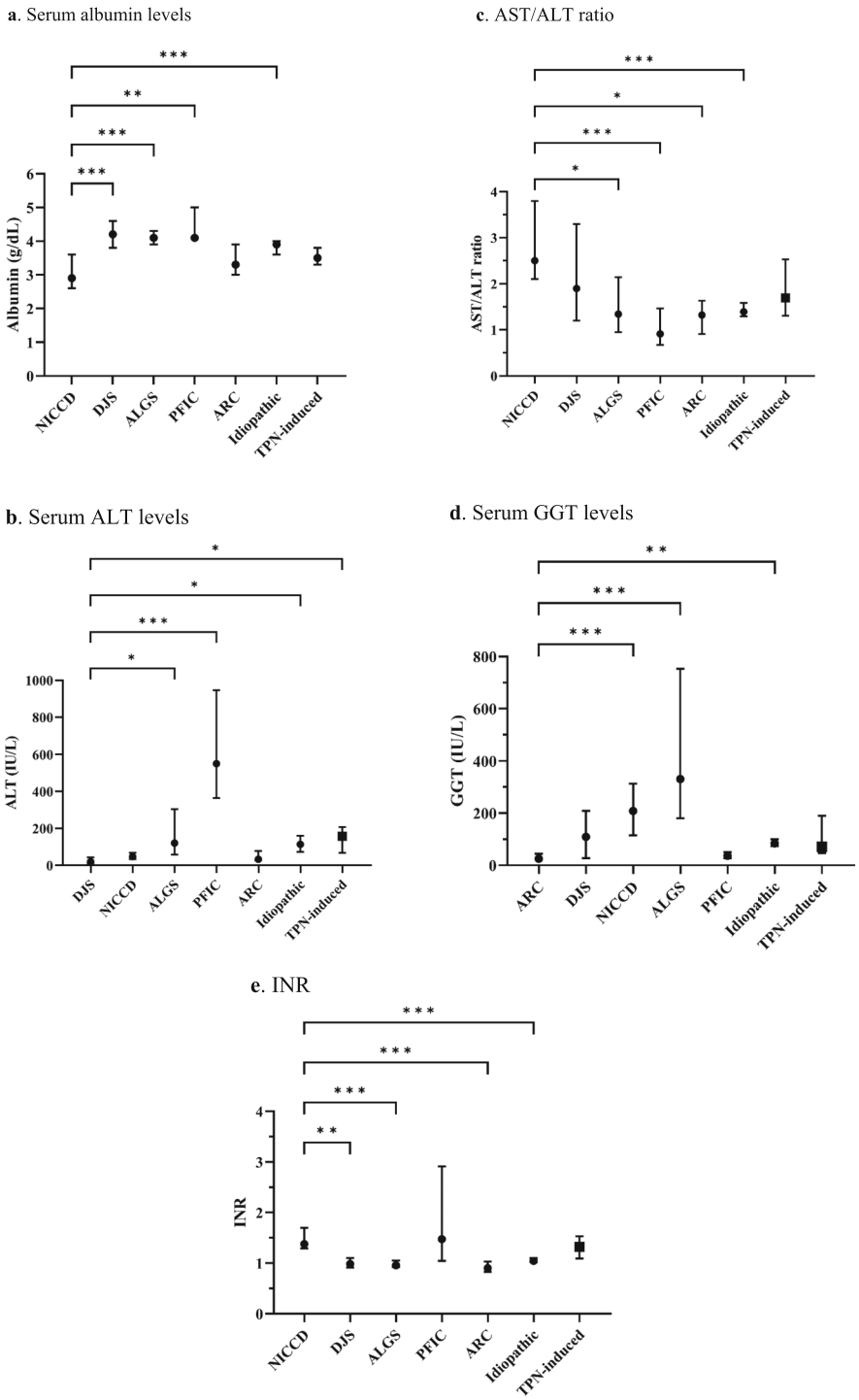

Figure 1 The differences in laboratory findings of the patients according to the final diagnosis. (a) Serum albumin levels; (b) serum ALT levels; (c) AST/ALT ratio; (d) serum GGT levels; (e) INR were compared between the groups of neonatal cholestasis. ALGS, Alagille syndrome; ALT, alanine aminotransferase; ARC, arthrogryposis-renal dysfunction-cholestasis; AST, aspartate aminotransferase; DJS, Dubin–Johnson syndrome; GGT, gamma glutamyl peptidase; INR, international normalized ratio; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; PFIC, progressive familial intrahepatic cholestasis; TPN, total parenteral nutrition. Kruskal–Wallis nonparametric one-way ANOVA test were performed. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

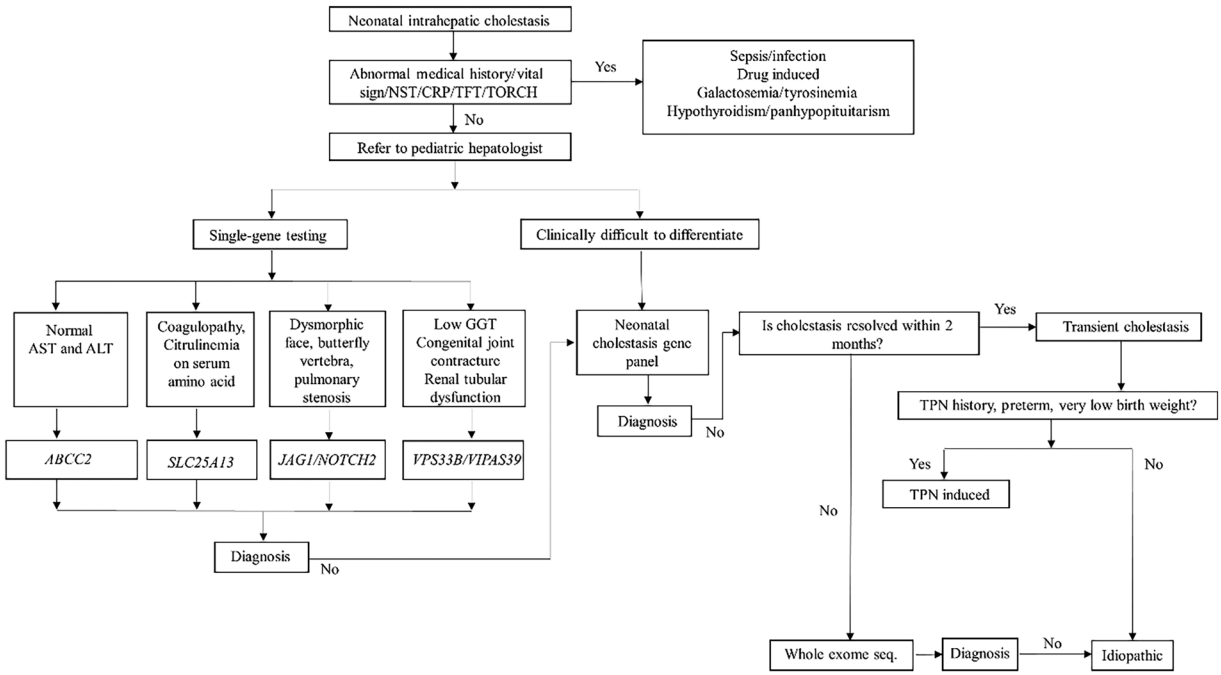

Figure 2 Diagnostic algorithm for neonatal intrahepatic cholestasis. ALT, alanine transferase; AST, aspartate transaminase; CRP, C-reactive protein; GGT, gamma glutamyl peptidase; NST, neonatal screening test; PS, pulmonary stenosis; sAA, serum amino acid; seq, sequencing; TFT, thyroid function test; TORCH, toxoplasmosis, rubella, cytomegalovirus, herpes simplex, and other viral agents; TPN, total parenteral nutrition.

Sanger sequencing and NGS has limitations in detecting large deletions and insertions in the causative genes of ALGS or NICCD, necessitating additional tests. In this study, multiplex ligation-dependent probe amplification for *JAG1* and PCR for the IVS16ins3kb mutation in *SLC25A13* were conducted, leading to diagnoses of NICCD in three patients. Other studies on NICCD have also used the confirmation of the IVS16ins3kb mutation for diagnosis.[25] In addition, in the case of persistent cholestasis that lacks a gene panel diagnosis, whole-exome sequencing should be considered. Several studies have shown that exome sequencing is a powerful tool in patients suspected of having monogenic disorders and can be used effectively in genetic neonatal cholestasis.[26,27] Indeed, one patient (subject 49) who showed neonatal cholestasis, chronic diarrhea, hyperammonemia, and hypoalbuminemia after birth was diagnosed with a *FARSA* mutation by whole-exome sequencing. Similar findings have previously been reported in another study.[28]

Among the 148 neonatal intrahepatic cholestasis patients in this study, 97 (65.5%) were male, indicating male predominance. Examining other neonatal cholestasis studies, in a cohort of 2171 patients, 1289 (59.4%) were male, and in another study with 154 patients, 92 (59.7%) were male, showing male predominance.[19,24] Multiple studies have described the characteristic laboratory findings of neonatal cholestasis. Consistent with our results, high GGT levels are associated with ALGS.[1,24] Additionally, previous studies reported that ALGS patients with the *NOTCH2* variant had significantly less facial, cardiac, and skeletal involvement than ALGS patients with the *JAG1* variant but similar kidney and ocular involvement, which is in accordance with our study.[29,30] In this study, low TP and Alb levels, high AST/ALT ratio, and high INR were identified in NICCD compared to those in other diseases. Low serum TP and Alb levels in NICCD have been reported in several studies.[11,31] Coagulopathy in NICCD was reported in a study in Japan, and decreased levels of vitamin K-dependent clotting factors such as fibrinogen and antithrombin have been reported as the causes of coagulopathy in NICCD.[32] There are many previous studies on the correlation between lower levels of AST and ALT and DJS,[14] which is consistent with our results. In addition, it has been reported that genetic analysis is essential for diagnosing DJS,[33–35] and based on clinical results in our study, DJS was included in single-gene sequencing in the diagnostic algorithm. Several genes associated with low GGT-inherited cholestasis are being actively studied, such as *ATP8B1*, *ABCB11*, *NR1H4*, *TJP2*, *LSR*, *USP53*, and *ABCC12*.[4–7,36,37] Because of the diverse causative genes for PFIC, PFIC was included in the gene panel of the diagnostic algorithm. One patient (subject 48) with neonatal cholestasis was genetically diagnosed with Kabuki syndrome by *KMT2D* sequencing, which is a rare presentation of bile duct paucity and low GGT cholestasis.[38]

With the increasing importance of the genetic portion in diagnosing neonatal cholestasis, well-established diagnostic algorithms with gene panels may elucidate the clinical significance of disease mutations, identify novel phenotypes, decrease the need for invasive procedures, and explore the mechanisms of neonatal cholestasis.[9,27] The proper application of modern broad-based gene panel sequencing is the latest guideline of the North American and European Society for Pediatric Gastroenterology, Hepatology, and Nutrition[1]; therefore, periodically updating the gene panel along with a well-established diagnostic algorithm for each region is required.

Here, we present an effective diagnostic algorithm for neonatal intrahepatic cholestasis. Deciding whether to perform single-gene

sequencing based solely on the patients' phenotype may lead to a delay in diagnosis in some cases. Therefore, our algorithm incorporates not only phenotype but also laboratory findings. However, it is important to note that different disease groups are prevalent in each country and region. Genetic neonatal cholestasis in North America is primarily caused by ALGS (*JAG1/NOTCH2*), alpha-1-antitrypsin deficiency (*SERPINA1*), cystic fibrosis (*CFTR*), DJS (*ABCC2*), and PFIC type II (*ABCB11*), while in Italy it is caused by ALGS (*JAG1/NOTCH2*), PFIC type II (*ABCB11*), and alpha-1-antitrypsin deficiency (*SERPINA1*).[16,19] In Japan and Taiwan, ALGS, PFIC, NICCD, and DJS[9,17] were the most common diagnoses of genetic neonatal cholestasis, which is similar to the findings of our study. Therefore, the diagnostic algorithm proposed in this study is applicable to East Asian populations. In addition, the algorithm presented in the study was developed based on tertiary hospital data and should be customized according to the capacity, resources, or budget at each center. Earlier utilization of a gene panel may facilitate timely diagnosis for treatable or severe diseases; however, it may decrease the diagnostic yield. The policy is suitable when resources are available. The main limitation of this study is its retrospective cohort design and single-center data. A multicenter or multinational study involving a greater number of patients with neonatal cholestasis is needed. Despite these limitations, this is the first study to analyze the clinical application of single-gene testing and NGS and to develop a diagnostic algorithm for neonatal intrahepatic cholestasis in Asia. Using our cost-effective diagnostic algorithm, it is possible to selectively perform single-gene testing rather than expensive gene panels for patients with suspected genetic neonatal cholestasis.

In conclusion, ALGS and NICCD were the most common diseases associated with genetic neonatal cholestasis in Korea. Single-gene testing and NGS are important and complementary tools for the diagnosis of genetic neonatal cholestasis. We developed a diagnostic algorithm for neonatal intrahepatic cholestasis that can be used in East Asia. Our study can also serve as a benchmark in developing a well-established diagnostic algorithm for each region.

Data availability statement. All data generated or analyzed during the current study are available in the clinVAR repository, https://www.ncbi.nlm.nih.gov/clinvar with accession numbers SCV002574966-SCV002574980.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Characteristics of the variants in the 69 patients who underwent neonatal cholestasis gene panel in this study.
Table S2. Comparison of characteristic between premature and non-premature patients.