# Molecular Genetic Dissection and Neonatal/Infantile Intrahepatic Cholestasis Using Targeted Next-Generation Sequencing

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**Objectives** To ascertain a molecular genetic diagnosis for subjects with neonatal/infantile intrahepatic cholestasis (NIIC) by the use of next-generation sequencing (NGS) and to perform a genotype-phenotype correlation.

**Study design** We recruited Japanese subjects with NIIC who had no definitive molecular genetic diagnosis. We developed a diagnostic custom panel of 18 genes, and the amplicon library was sequenced via NGS. We then compared clinical data between the molecular genetically confirmed subjects with NIIC.

**Results** We analyzed 109 patients with NIIC ("genetic cholestasis," 31 subjects; "unknown with complications" such as prematurity, 46 subjects; "unknown without complications," 32 subjects), and a molecular genetic diagnosis was made for 28 subjects (26%). The rate of positive molecular genetic diagnosis in each category was 22 of 31 (71%) for the "genetic cholestasis" group, 2 of 46 (4.3%) for the "unknown with complications" group, and 4 of 32 (12.5%) for the "unknown without complications" group. The grouping of the molecular diagnoses in the group with genetic cholestasis was as follows: 12 with Alagille syndrome, 5 with neonatal Dubin-Johnson syndrome, 5 with neonatal intrahepatic cholestasis caused by citrin deficiency, and 6 with progressive familial intrahepatic cholestasis or benign recurrent intrahepatic cholestasis with low gamma-glutamyl transpeptidase levels. Several clinical datasets, including age of onset, direct bilirubin, and aminotransferases, were significantly different between the disorders confirmed using molecular genetic diagnosis.

**Conclusion** Targeted NGS can be used for molecular genetic diagnosis in subjects with NIIC. Clinical diagnosis should be accordingly redefined in the view of molecular genetic findings. *(J Pediatr 2016;171:171-7)*.

The etiologic diversity of neonatal/infantile cholestasis has been described previously.[1,2] The most commonly identifiable etiologies are biliary atresia (25%-35%), genetic intrahepatic cholestasis (25%), and metabolic diseases (20%).[2,3] Recent advances in the understanding of the molecular basis of cholestatic syndromes have enabled the classification of these syndromes and have offered an opportunity for the development of diagnostic methods that take into account the genetic makeup of neonatal/infantile intrahepatic cholestasis (NIIC).[1-3] Alagille syndrome (ALGS), progressive familial intrahepatic cholestasis (PFIC), neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and other conditions were distinguished from idiopathic neonatal hepatitis between the early 1970s and the 2000s.[1,2] In addition, in the past 2 decades, a wide range of disease-causing genes underlying the pathogenesis of NIIC has been identified.[1,4] The high genetic heterogeneity and variability of NIIC make it difficult to survey for pathogenic variants in clinical practice, and molecular genetic diagnosis challenging.

Next-generation sequencing (NGS) technologies have revolutionized genomic and clinical genetic research.[5,6] NGS offers comprehensive sequencing of multiple known causative or associated genes in highly heterogeneous diseases.[5] Here, we constructed an NIIC gene panel that included 18 candidate genes. In this study we aimed to use this panel and NGS to ascertain the molecular genetic diagnosis of subjects with NIIC. We then compared clinical and laboratory findings for patients in whom a molecular genetic diagnosis was made to obtain a comprehensive understanding of NIIC.

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| ALGS | Alagille syndrome | IR 4.0 | Ion Reporter 4.0 |
| ALT | Alanine aminotransferase | MAF | Minor allele frequency |
| AR | Autosomal-recessive inheritance | MLPA | Multiplex ligation-dependent probe amplification |
| AST | Aspartate aminotransferase | NGS | Next-generation sequencing |
| BRIC | Benign recurrent intrahepatic cholestasis | NICCD | Neonatal intrahepatic cholestasis caused by citrin deficiency |
| CNV | Copy number variation | NIIC | Neonatal/infantile intrahepatic cholestasis |
| D.Bil | Direct bilirubin | PFIC | Progressive familial intrahepatic cholestasis |
| DJS | Dubin-Johnson syndrome | T.Bil | Total bilirubin |
| GGT | Gamma glutamyl transpeptidase | | |
| HGVB | Human Genetic Variation Browser | | |
| Ion PGM | Ion Torrent Personal Genome Machine | | |

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## Methods

Written informed consent was obtained from the parents. Experimental protocols were approved by the Ethical Committee for the Study of Human Gene Analysis at Nagoya City University Graduate School of Medical Sciences (approval number 150).

Serum-conjugated hyperbilirubinemia is the most common marker of cholestasis.[7] Here, cholestasis was defined as follows: (1) a serum direct bilirubin (D.Bil) level of >1.0 mg/dL, if the total bilirubin (T.Bil) was <5.0 mg/dL; or (2) a serum D.Bil level of more than 20% of the T.Bil level if the T.Bil level was >5.0 mg/dL.[3,7] From April 2013 to August 2015, we recruited Japanese subjects with NIIC with the following entry criteria: (1) cholestasis; (2) onset <12 months of age; (3) date of birth between January 2010 and December 2014; and (4) no definitive molecular diagnosis previously. The exclusion criteria were extrahepatic cholestasis, such as biliary structural abnormality, or chromosomal abnormality.

### Clinical Diagnosis of NIICs and Collection of the Laboratory Findings

Clinical diagnosis of all the subjects was confirmed by reviewing the available clinical and laboratory records at the time of registration. We classified the recruited subjects into 3 subcategories: (1) "genetic cholestasis" (ie, subjects who were clinically diagnosed with known genetic cholestasis syndromes such as ALGS, PFIC, NICCD, and Dubin-Johnson syndrome [DJS]); (2) "unknown with complications" (ie, subjects with no definitive clinically identified etiology, although they showed potential cholestasis-causative complications such as prematurity, infections, and metabolic or hormonal system abnormalities); and (3) "unknown without complications" (ie, subjects with no definitive etiology or no potentially cholestasis-causative complications).

ALGS with autosomal-dominant inheritance was diagnosed according to the classical definition of the presence of 3 of 5 major clinical criteria.[8] Diagnosis of neonatal DJS with autosomal-recessive inheritance (AR) was determined by the presence of a brown/black liver and a normal value of aspartate aminotransferase/alanine aminotransferase (AST/ALT).[9-11] Diagnosis of NICCD with AR was based on clinical suspicion, supported by laboratory evidence including serum amino acid profiles or liver histology.[12] The clinical diagnosis of PFIC/benign recurrent intrahepatic cholestasis (BRIC) with AR was based on the clinical history of infantile age, serologic low/normal gamma glutamyl transpeptidase (GGT) level, and histologic features.[13-15] For laboratory findings at presentation, we adopted the measurements available at the same period as the greatest D.Bil value obtained.

### Targeted Genes, Amplicon Library Preparation, and NGS

An amplicon library of the target exons and flanking sequences was prepared with the use of an Ion AmpliSeq Custom Panel (Life Technologies, Carlsbad, California). This custom NIIC panel (panel ID: IAD34922) contained the *JAG1*, *NOTCH2*, *ABCC2*, *SLC25A13*, *ATP8B1*, *ABCB11*, *ABCB4*, *TJP2*, *HSD3B7*, *AKR1D1*, *CYP7B1*, *VPS33B*, *BAAT*, *EPHX1*, *SLC10A1*, *ABCB1*, *SLC4A2*, and *SLCO1A2* genes. The first 14 genes listed are known intrahepatic cholestasis disease-causing genes,[1,4] and the last 4 are potential candidate genes encoding proteins that play a role in bile acid transport. The genes on the panel were selected on the basis of the prevalence of the variants in the Japanese population, and thus customized for Japanese patients with NIIC. The number of exons, amplicons, and total targeted bases were 348, 546, and 52 795 bases, respectively. This NIIC panel allowed theoretical coverage of 98.5% of the targeted sequences (**Table I**; available at www.jpeds.com). Genomic DNA was extracted from peripheral blood using the QIAamp Blood Midi Kit (QIAGEN, Hilden, Germany). The library was constructed by use of the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Carlsbad, California). Emulsion polymerase chain reaction was carried out using the Ion OneTouch 2 system (Life Technologies). NGS was performed with the Ion Torrent Personal Genome Machine (Ion PGM) system (Life Technologies).

### Sequence Data Analysis Using Bioinformatics and Validation Analysis

Sequence data analysis pipelines were established with use of the workflow in CLC Genomics Workbench 7.0 (CLC bio, Aarhus, Denmark) and Ion Reporter 4.0 (IR 4.0; Life Technologies, Carlsbad, California). IR 4.0 could potentially call a copy number variation (CNV). The Human Gene Mutation Database in January 2015 (http://www.hgmd.org/; BioBase, Waltham, Massachusetts), Sorting Intolerant From Tolerant (http://sift.jcvi.org/; J. Craig Venter Institute, Rockville, Maryland), Polymorphism Phenotyping-2 (http://genetics.bwh.harvard.edu/pph2/; Harvard Medical School, Boston, Massachusetts, USA), and Human Splicing Finder ver 3.0 (http://www.umd.be/HSF3/; Aix-Marseille University, Marseille, France) were used with computational predictive programs.[16-18] Minor allele frequency (MAF) was referred to the Japanese data set of the Human Genetic Variation Browser (HGVB; http://www.genome.med.kyoto-u.ac.jp/SnpDB; Kyoto University, Kyoto, Japan) that was released in November 2013 and to the Exome Aggregation Consortium (http://exac.broadinstitute.org; Cambridge, Massachusetts, accessed August 2015). The American College of Medical Genetics and Genomics interpretation guidelines were followed in assessing the pathogenicity of the detected variants.[19] The nomenclature of identified variants was assigned according to the guidelines of the Human Genome Variation Society version 2 (http://www.hgvs.org/mutnomen/; Human Genome Variation Society, Melbourne, Australia). Chromosomal coordinates were assigned according to the GRCh37/hg19 assembly. All candidate variants were validated by conventional Sanger sequencing, and CNVs were confirmed by multiplex ligation-dependent probe amplification (MLPA) analysis.[20]