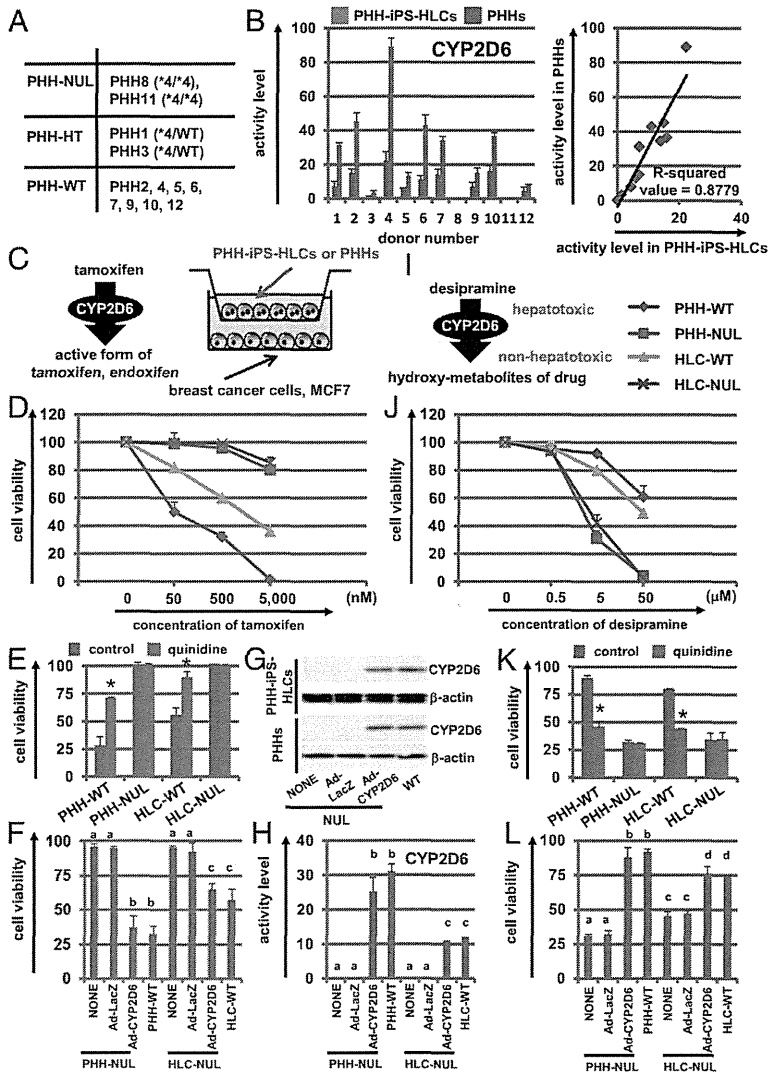

Fig. 4. The interindividual differences in CYP2D6 metabolism capacity and drug responsiveness induced by SNPs in CYP2D6 are reproduced in the PHH-iPS-HLCs. (*A*) SNPs (CYP2D6*3, *4, *5, *6, *7, *8, *16, and *21) in the CYP2D6 gene were analyzed. (*B*) The CYP2D6 activity levels in PHH-iPS-HLCs and PHHs were measured by LC-MS/MS analysis. (*C*) The pharmacological activity of tamoxifen-dependent conversion to its metabolite, endoxifen, by the CYP2D6. The coculture system of breast cancer cells (MCF-7 cells) and the PHH-iPS-HLCs are illustrated. (*D*) The cell viability of MCF-7 cells was assessed after 72-h exposure to different concentrations of tamoxifen. (*E*) The cell viability of MCF-7 cells, which were cocultured with PHH-WT, PHH-NUL, HLC-WT, and HLC-NUL, was assessed after 72-h exposure to 500 nM of tamoxifen in the presence or absence of 3 nM quinidine (a CYP2D6 inhibitor). (*F*) The cell viability of MCF-7 cells cocultured with Ad-CYP2D6-transduced PHH-NUL and HLC-NUL was examined after 72-h exposure to 500 nM of tamoxifen. (*G* and *H*) The CYP2D6 expression (*G*) and activity (*H*) levels in Ad-CYP2D6-transduced PHH-NUL and HLC-NUL were examined by Western blotting and LC-MS/MS analysis. (*I*) The detoxification of desipramine-dependent conversion to its conjugated form by the CYP2D6. (*J*) The cell viability of PHH-WT, PHH-NUL, HLC-WT, and HLC-NUL was assessed after 24-h exposure to different concentrations of desipramine. (*K*) The cell viability of the PHH-WT and HLC-WT was assessed after 24-h exposure to 5 μM of desipramine in the presence or absence of 5 μM of quinidine (a CYP2D6 inhibitor). (*L*) The cell viability of the Ad-CYP2D6-transduced PHH-NUL and HLC-NUL was examined after 24-h exposure to 5 μM of desipramine. The cell viability was expressed as a percentage of that in the cells treated with only solvent. Data represent the mean ± SD from three independent experiments. In *E* and *K*, Student *t* test indicated that the cell viability in the "control" was significantly higher than that in the "quinidine" group ($P < 0.01$). In *F*, *H*, and *L*, statistical significance was evaluated by ANOVA followed by Bonferroni post hoc tests to compare all groups. Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

in this study have the CYP2D6 poor metabolizer genotypes (CYP2D6 *3, *4, *5, *6, *7, *8, *16, and *21) (5). PHH8 and -11 have CYP2D6*4 (null allele), whereas the others have a wild type (WT) or hetero allele (*SI Appendix*, Table S3 and Fig. 4*A*). Consistent with this finding, the PHH8/11-iPS-HLCs also have CYP2D6*4, whereas the others have a wild type or hetero allele. As expected, the CYP2D6 activity levels in the PHH8/11 (PHH-NUL) and PHH8/11-iPS-HLC (HLC-NUL) were significantly lower than those in the PHH-WT and HLC-WT, respectively (Fig. 4*B*). The pharmacological activity of tamoxifen, which is the most widely used agent for patients with breast cancer, is dependent on its conversion to its metabolite, endoxifen, by the CYP2D6 (Fig. 4*C*). To examine whether the pharmacological activity of tamoxifen could be predicted by using PHHs and HLCs that have either the null type CYP2D6*4 allele or wild-type CYP2D6 allele, the breast cancer cell line MCF7 was cocultured with PHHs or HLCs, and then the cells were treated with tamoxifen (Fig. 4*D*). The cell viability of MCF7 cells cocultured with PHHs-NUL or HLCs-NUL was significantly higher than that of MCF7 cells cocultured with PHHs-WT or HLCs-WT. The decrease in cell viability of MCF7 cells cocultured with PHHs-WT or HLCs-WT was rescued by treatment with a CYP2D6 inhibitor, quinidine (Fig. 4*E*). We also confirmed that the cell viability of MCF7 cells cocultured with PHHs-NUL or HLCs-NUL was decreased by CYP2D6 overexpression in the PHHs-NUL or HLCs-NUL (Fig. 4*F*). Note that the expression (Fig. 4*G*) and activity (Fig. 4*H*) levels of CYP2D6 in CYP2D6-expressing adenovirus vector (Ad-CYP2D6)-transduced PHHs-NUL or HLCs-NUL were comparable to those of PHHs-WT or HLCs-WT. These results indicated that the PHHs-WT and HLCs-WT could more efficiently metabolize tamoxifen than the PHHs-NUL and HLCs-NUL, respectively, and thereby induced higher toxicity in MCF7 cells. Similar results were obtained with the other breast cancer cell line, T-47D (*SI Appendix*, Fig. S8 *A–D*). Next, we examined whether the CYP2D6-mediated drug-induced hepatotoxicity could be predicted by using PHHs and HLCs having either a null type CYP2D6*4 allele or wild-type CYP2D6 allele. PHHs and HLCs were treated with desipramine, which is known to cause hepatotoxicity (Fig. 4*I*) (14). The cell viability of PHHs-NUL and HLCs-NUL was significantly lower than that of PHHs-WT and HLCs-WT (Fig. 4*J*). The cell viability of the PHHs-WT or HLCs-WT was decreased by treatment with a CYP2D6 inhibitor, quinidine (Fig. 4*K*). We also confirmed that the decrease in the cell viability of the PHHs-NUL or HLCs-NUL was rescued by CYP2D6 overexpression in the PHHs-NUL or HLCs-NUL (Fig. 4*L*). Similar

results were obtained with the other hepatotoxic drug, perhexiline (*SI Appendix*, Fig. S8 *E–H*). These results indicated that the PHHs-WT and HLCs-WT could more efficiently metabolize imipramine and thereby reduce toxicity compared with the PHHs-NUL and HLCs-NUL. Taken together, our findings showed that the interindividual differences in CYP metabolism capacity and drug responsiveness, which are prescribed by an SNP in genes encoding CYPs, were also reproduced in the PHH-iPS-HLCs.

Discussion

The purpose of this study was to examine whether the individual HLCs could reproduce the hepatic function of individual PHHs. A Yamanaka 4 factor-expressing SeV vector was used in this study to generate integration-free human iPSCs from PHHs. It is known that SeV vectors can express exogenous genes without chromosomal insertion, because these vectors replicate their genomes exclusively in the cytoplasm (15). To examine the different cellular phenotypes associated with SNPs in human iPSC derivatives, the use of integration-free human iPSCs is essential.

We found that the CYP activity levels of the PHH-iPS-HLCs reflected those of parent PHHs, as shown in Fig. 3 *A–C*. There were few interindividual differences in the ratio of CYP expression levels in the PHH-iPS-HLCs to those in PHHs (*SI Appendix*, Fig. S5). Together, these results suggest that it is possible to predict the individual CYP activity levels through analysis of the CYP activity levels of the PHH-iPS-HLCs. In the future, it will be necessary to confirm these results in skin or blood cell-derived iPSCs as well as PHH-iPSCs, although donor-matched PHHs and blood cells (or skin cells) are difficult to obtain. In addition, the comparison of hepatic functions between genetically identical PHHs and PHH-iPS-HLCs (Fig. 3 *A–C*) would enable us to accurately ascertain whether the HLCs exhibit sufficient hepatic function to be a suitable substitute for PHHs in the early phase of pharmaceutical development. Because the drug responsiveness of the individual HLCs reflected that of individual PHHs (Fig. 3 *E* and *F*), it might be possible to perform personalized drug therapy following drug screening using a patient's HLCs. However, the R-squared values of the individual CYP activities differed from each other (Fig. 3 *A–C*), suggesting that the activity levels of some CYPs are largely influenced not only by genetic information but also by environmental factors, such as dietary or smoking habits.

The interindividual differences of CYP2D6 metabolism capacity and drug responsiveness that were prescribed by SNP in genes encoding CYP2D6 were reproduced in the PHH-iPS-HLCs (Fig. 4). It was impossible to perform drug screening in the human hepatocytes derived from a donor with rare SNPs because these hepatocytes could not be obtained. However, because human iPSCs can be generated from such donors with rare SNPs, the CYP metabolism capacity and drug responsiveness of these donors might be possible to predict. Further, it would also be possible to identify the novel SNP responsible for an unexpected hepatotoxicity by using the HLCs in which whole genome sequences are known. We thus believe that the HLCs will be a powerful tool not only for accurate and efficient drug development but also for personalized drug therapy.

Experimental Procedures

DNA Microarray. Total RNA was prepared from the PHH9-iPSCs, PHH9-iPS-HLCs, PHH9, and human hepatocellular carcinoma cell lines by using an RNeasy Mini kit. A pool of three independent samples was used in this study. cRNA amplifying, labeling, hybridizing, and analyzing were performed at Miltenyi Biotec. The Gene Expression Omnibus (GEO) accession no. for the microarray analysis is GSE61287.

Flow Cytometry. Single-cell suspensions of human iPSC-derived cells were fixed with 2% (vol/vol) paraformaldehyde (PFA) for 20 min, and then incubated with the primary antibody (described in *SI Appendix*, Table S1), followed by the secondary antibody (described in *SI Appendix*, Table S2). In case of the intracellular staining, the Permeabilization Buffer (eBioscience) was used to create holes in the membrane thereby allowing the antibodies to enter the cell effectively. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

ACKNOWLEDGMENTS. We thank Yasuko Hagihara, Natsumi Mimura, and Shigemi Isoyama for their excellent technical support. H.M. and K.K. were supported by grants from the Ministry of Health, Labor, and Welfare. H.M. was also supported by the Project for Technological Development, Research Center Network for Realization of Regenerative Medicine of the Japan Science and Technology Agency and by the Uehara Memorial Foundation. F.S. was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. K.T. and Y.N. were supported by a grant-in-aid for the Japan Society for the Promotion of Science Fellows.

1. Takayama K, et al. (2012) Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction. *Mol Ther* 20(1):127–137.
2. Medine CN, et al. (2013) Developing high-fidelity hepatotoxicity models from pluripotent stem cells. *Stem Cells Transl Med* 2(7):505–509.
3. Ingelman-Sundberg M (2004) Pharmacogenetics of cytochrome P450 and its applications in drug therapy: The past, present and future. *Trends Pharmacol Sci* 25(4): 193–200.
4. Ingelman-Sundberg M (2001) Genetic susceptibility to adverse effects of drugs and environmental toxicants. The role of the CYP family of enzymes. *Mutat Res* 482(1-2): 11–19.
5. Zhou SF (2009) Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. *Clin Pharmacokinet* 48(11):689–723.
6. Borges S, et al. (2006) Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: Implication for optimization of breast cancer treatment. *Clin Pharmacol Ther* 80(1):61–74.
7. Bakke OM, Manocchia M, de Abajo F, Kaitin KI, Lasagna L (1995) Drug safety discontinuations in the United Kingdom, the United States, and Spain from 1974 through 1993: A regulatory perspective. *Clin Pharmacol Ther* 58(1):108–117.
8. Lin T, et al. (2009) A chemical platform for improved induction of human iPSCs. *Nat Methods* 6(11):805–808.
9. Polo JM, et al. (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol* 28(8):848–855.
10. Takayama K, et al. (2013) Long-term self-renewal of human ES/iPS-derived hepatoblast-like cells on human laminin 111-coated dishes. *Stem Cell Reports* 1(4):322–335.
11. McDonald MG, Rettie AE (2007) Sequential metabolism and bioactivation of the hepatotoxin benzbromarone: Formation of glutathione adducts from a catechol intermediate. *Chem Res Toxicol* 20(12):1833–1842.
12. Kaufmann P, et al. (2005) Mechanisms of benzarone and benzbromarone-induced hepatic toxicity. *Hepatology* 41(4):925–935.
13. Ingelman-Sundberg M (2005) Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): Clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J* 5(1):6–13.
14. Spina E, et al. (1997) Relationship between plasma desipramine levels, CYP2D6 phenotype and clinical response to desipramine: A prospective study. *Eur J Clin Pharmacol* 51(5):395–398.
15. Nishimura K, et al. (2011) Development of defective and persistent Sendai virus vector: A unique gene delivery/expression system ideal for cell reprogramming. *J Biol Chem* 286(6):4760–4771.

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2015) **57**, 113–118 doi: 10.1111/ped.12449

Original Article

Scoring system for the prediction of severe acute pancreatitis in children

Mitsuyoshi Suzuki,[1] Nobutomo Saito,[1] Nakayuki Naritaka,[1] Satoshi Nakano,[1] Kei Minowa,[1] Yuka Honda,[1] Yoshikazu Ohtsuka,[1] Atsuyuki Yamataka[2] and Toshiaki Shimizu[1]

Departments of [1]Pediatrics and [2]Pediatric General and Urogenital Surgery, Juntendo University School of Medicine, Tokyo, Japan

Abstract ***Background***: The lack of an accurate scoring system for pediatric acute pancreatitis could cause delays in appropriate clinical management and increase the risk of progressive life-threatening complications. We investigated a modified Ministry of Health, Labour and Welfare of Japan (JPN) scoring system that uses pediatric systemic inflammatory response syndrome (SIRS) score, age, and weight to establish a more useful scoring system for children.

Methods: A retrospective chart review was conducted of pediatric patients with acute pancreatitis who were admitted to Juntendo University Hospital between 1985 and 2011. The sensitivity, specificity, and positive and negative predictive values of the pediatric JPN scoring system were calculated and then compared with those of previously developed scoring systems.

Results: The patient group consisted of 145 patients (88 girls, 57 boys). The pediatric JPN score had greater sensitivity (80%) than the Ranson (60%), modified Glasgow (50%), and DeBanto (60%) scores. The specificity was 96% for the pediatric JPN score, 94% for the Ranson score, 99% for the modified Glasgow score, and 86% for the DeBanto score.

Conclusion: The pediatric JPN score can be used to predict severe acute pancreatitis during the initial medical assessment.

Key words guideline, Ministry of Health, Labour and Welfare of Japan (JPN) scoring system, pediatrics, severe acute pancreatitis, severity assessment.

In 2002, DeBanto *et al.* were the first to suggest a scoring system for predicting the severity of acute pancreatitis (AP) in children.[1] This system was modified from the Ranson[2,3] and Glasgow systems[4] and consists of the following eight parameters: age (<7 years old); weight (<23 kg); white blood cell count at admission (>18 500 cells/μL); lactate dehydrogenase (LDH) at admission (>2000 U/L); 48 h trough Ca^{2+} (<8.3 mg/dL); 48 h trough albumin (<2.6 g/dL); 48 h fluid sequestration (>75 mL/kg per 48 h); and 48 h rise in blood urea nitrogen (BUN; >5 mg/dL). Patients who met three or more of these criteria were predicted to have a severe outcome (Table 1). This scoring system, however, is not exact for Asian children.[6] Recently, Lautz *et al.* reported that the Ranson, modified Glasgow, and DeBanto pediatric scores have limited ability to predict AP severity in children and adolescents in the USA.[7] Assessment of severity at the initial medical examination plays an important role in providing adequate early treatment and transferring patients to a medical facility equipped to treat severe AP. The lack of an accurate scoring system for pediatric AP, however, could cause delays in appropriate clinical management and increase the risk of progressive life-threatening complications.

In 1990, the AP severity scoring system of the Ministry of Health, Labour and Welfare of Japan (JPN score) was developed.[8] The scoring system was partially revised in 1999 and revised again to the current version in 2008 (Table 1).[5,8] The current criteria consist of nine prognostic factors and/or computed tomography (CT) grades based on contrast-enhanced CT. This system, however, does not reflect the characteristics of children and adolescents. The JPN score criteria (2008 version) include two parameters, systemic inflammatory response syndrome (SIRS) score and age ≥70 years, that make the system inappropriate for use in children.[5] Similarly, the Ranson and modified Glasgow scores contain a parameter concerning age (≥55 years) that is not appropriate for pediatric patients.[3,4] In the present study, we investigated a modified JPN score that reflects pediatric SIRS score, age, and weight to establish a more useful scoring system for children.

Methods

The current study was a retrospective chart review of all children and adolescents (<18 years of age) with an ICD-9 code of 577.0 or an ICD-10 code of K85 who were admitted to the Pediatric or Pediatric Surgery Department of Juntendo University Hospital

Correspondence: Mitsuyoshi Suzuki, MD PhD, Department of Pediatrics, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Email: msuzuki@juntendo.ac.jp

Received 8 September 2013; revised 17 February 2014; accepted 3 July 2014.

Table 1 Comparison of parameters of three scoring systems

Parameter	Pediatric JPN scoring system	JPN scoring system (2008 version)[5]	Parameter	DeBanto score[1]
1	BE ≤ −3 mEq or shock (Table 2 lists SBP depending on age)	BE ≤ −3 mEq or shock (SBP ≤80 mmHg)		
2	PaO_2 ≤60 mmHg (room air) or pulmonary insufficiency (ventilation required)	PaO_2 ≤60 mmHg (room air) or pulmonary insufficiency (ventilation required)		
3	BUN ≥40 mg/dL (or Cr ≥2 mg/dL) or urine volume <0.5 mL/kg/h even after fluid resuscitation	BUN ≥40 mg/dL (or Cr ≥2 mg/dL) or urine volume <400 mL/day even after fluid resuscitation	1 2	48 h rise in BUN >5 mg/dL 48 h fluid sequestration <75 mL/kg/48 h
4	LDH ≥2 × above upper limit (Age-adjusted value)	LDH ≥2 × above upper limit	3	Admission LDH >2000 U/L
5	Platelet count ≤1 × 10^5/mm^3	Platelet count ≤1 × 10^5/mm^3		
6	Ca ≤7.5 mg/dL	Ca ≤7.5 mg/dL	4	48 h trough Ca <8.3 mg/dL
7	CRP ≥15 mg/dL	CRP ≥15 mg/dL		
8	Pediatric SIRS score ≥3	SIRS score ≥3	5	Admission white blood cell count >18 500 cell/μL
9	Age <7 years and/or weight <23 kg	Age ≥70 years	6 7 8	Age <7 years Weight <23 kg 48 h trough albumin <2.6 g/dL

BE, base excess; BUN, blood urea nitrogen; Ca, calcium; Cr, creatinine; CRP, C-reactive protein; JPN, Ministry of Health, Labour and Welfare of Japan; LDH, lactate dehydrogenase; PaO_2, partial pressure of arterial oxygen; SBP, systolic blood pressure; SIRS, systemic inflammatory response syndrome.

between 1985 and 2011. This study was approved by the Juntendo University Institutional Review Board. The diagnosis of AP was confirmed on elevation of serum amylase or lipase more than twofold the upper limit of normal, clinical signs and symptoms, and/or imaging.

Based on the JPN scoring system (2008 version), the pediatric JPN scoring system was developed using pediatric SIRS score (Tables 2,3)[9] and the parameters of age <7 years old and/or weight <23 kg, as reported by DeBanto *et al.*[1] The parameters of the pediatric JPN score were as follows: (i) base excess (BE) ≤−3 mEq or shock (systolic blood pressure cut-offs according to age group; Table 3); (ii) PaO_2 ≤60 mmHg (room air) or respiratory failure; (iii) BUN ≥40 mg/dL (or creatinine [Cr] ≥2.0 mg/dL) or oliguria (<0.5 mL/kg per h); (iv) LDH ≥2 × upper limit; (v) platelet count ≤1 × 10^5/mm^3; (vi) calcium (Ca) ≤7.5 mg/dL; (vii) C-reactive protein (CRP) ≥15 mg/dL; (viii) number of positive measures in pediatric SIRS score ≥3; and (ix) age <7 years old or/and weight <23 kg. The cut-off for predicting a severe outcome was set at three criteria. A comparison of the parameters of the present system, the JPN scoring system (2008 version), and the DeBanto system is shown in Table 1. To identify the severe patients who were initially diagnosed as having mild pancreatitis, the severity was determined using data from 72 h after the onset of pancreatitis.

An episode of pancreatitis was considered to be clinically severe if the patient died; had pancreatic surgery; developed a pseudocyst, abscess, or infected necrosis; or met the Atlanta criteria (1992 version) for organ dysfunction (systolic blood pressure, 90 mmHg; PaO_2, 60 mmHg; creatinine, 2 mg/dL; or gastrointestinal bleeding, 500 mL/24 h).[1,10] The clinical and laboratory

Table 2 Pediatric SIRS score[9]

- Core temperature >38.5°C or 36.0°C
- Tachycardia, defined as mean heart rate >2 SD above normal for age; or for children <1 year old, bradycardia, defined as mean heart rate in the <10th percentile for age.
- Mean respiratory rate >2 SD above normal for age.
- Leukocyte count elevated or decreased for age or >10% immature neutrophils.

SIRS, systemic inflammatory response syndrome.

Table 3 Age-specific vital signs and laboratory variables[9]

Age group	Tachycardia (beats/min)	Bradycardia (beats/min)	Respiratory rate (breaths/min)	Leukocyte count (×10^3/mm^3)	Systolic blood pressure (mmHg)
0 day–1 week	>180	<100	>50	>34	<65
1 week–1 month	>180	<100	>40	>19.5 or <5	<75
1 month–1 year	>180	<90	>34	>17.5 or <5	<100
2–5 years	>140	NA	>22	>15.5 or <6	<94
6–12 years	>130	NA	>18	>13.5 or <4.5	<105
13–18 years	>110	NA	>14	>11 or <4.5	<117

Lower values for heart rate, leukocyte count, and systolic blood pressure are for the 5th percentile, and upper values for the heart rate, respiration rate, or leukocyte count are for the 95th percentile. NA, not available.

Table 4 Demographic characteristics and etiology

	Mild AP	Severe AP
N	135	10
Age (years), mean ± SE (range)	7.38 ± 0.03 (0.8–17)	6.40 ± 0.42 (2–13)
M : F	51:84	6:4
Etiology		
1. Congenital anomalies of the pancreaticobiliary system		
• Choledochal cyst and/or abnormal union of the pancreaticobiliary junction	46 (34.1)	1 (10.0)
• Pancreas divisum	9 (6.7)	1 (10.0)
• Gallstones	9 (6.7)	0
• Others	13 (9.6)	0
2. Drugs	16 (11.9)	3 (30.0)
3. Trauma	14 (10.3)	0
4. Multisystem disease	8 (5.9)	5 (50.0)
5. Infections	5 (3.7)	0
6. Familial	3 (2.2)	0
7. Others	12 (8.9)	0

AP, acute pancreatitis.

factors used in the Ranson, modified Glasgow, DeBanto, and pediatric JPN scores were compared between patients with mild and severe AP. Finally, using a score of three or more positive parameters as a positive result, the sensitivity, specificity, and positive and negative predictive values were calculated.

Statistical analysis

The results are expressed as mean ± SE. Unpaired Mann–Whitney *U*-test and Fisher's exact test were used to identify significant changes, as appropriate. Receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC) was calculated to compare the overall accuracy of the different scoring systems. The AUC ± SE was calculated under non-parametric assumptions. All *P*-values were two-tailed, and $P < 0.05$ was considered statistically significant. Statistical analysis was done using SPSS version 18.0 (IBM, Tokyo, Japan).

Results

Patient demographics and AP etiology

The patient group consisted of 145 patients (88 girls, 57 boys) aged 0.8–17 years (mean age, 7.3 years). Of the 145 AP patients, 79 (54.5%) had congenital anomalies of the pancreaticobiliary system. Of these 79 children, common bile duct dilatation and/or abnormal union of the pancreaticobiliary junction was present in 47 children. Nineteen children had drug-induced AP (13.1%), and 14 children had trauma-induced AP (9.7%). Thirteen children had multisystem disease (9.0%), five had infections (3.4%), and 12 had AP of unknown etiology (8.3%). The demographic data for both the mild and severe groups according to the Atlanta criteria are given in Table 4.

Criteria for severity by Atlanta criteria

According to the Atlanta criteria, 10 children had severe pancreatitis, including two who died. The total number of criteria met was 27, because four patients met one criterion, two patients met two criteria, two patients met three criteria, one patient met four criteria, and one patient met nine criteria (Table 5).

Clinical and laboratory parameters of pediatric JPN score

The results of the statistical analysis between mild and severe cases of AP for 12 individual clinical and laboratory parameters are listed in Table 6. The parameters of BE ($P \leq 0.05$), BUN ($P \leq 0.01$), LDH ($P \leq 0.01$), and CRP ($P \leq 0.01$) achieved a statistically significant difference between mild and severe AP, while no significant difference was found for platelet count, serum calcium level, age or weight. The incidence of shock ($P \leq 0.01$), pulmonary insufficiency ($P \leq 0.01$), oliguria ($P \leq 0.01$), and pediatric SIRS score ≥3 ($P \leq 0.01$) was significantly higher in severe AP than mild AP.

Outcome: Pediatric JPN score compared to Atlanta criteria

The consistency between pediatric JPN score and Atlanta criteria was examined. Severe outcome occurred in 1.5% (2/132) of patients with 0–2 points, 50.0% (5/10) of patients with 3–4 points, 100% (2/2) of patients with 5–6 points, and 100% (1/1) in the patient with 7–8 points. The mortality of severe AP identified using the Atlanta criteria was 20% (2/10); the pediatric JPN scores of these patients were 5 and 7 points.

Table 5 Criteria for severity on Atlanta criteria

Criteria for severity	No. patients (%)
1. Death	2 (20)
2. Surgery on pancreas	1 (10)
3. Shock	8 (80)
4. Pulmonary insufficiency	4 (40)
5. Renal failure	6 (60)
6. Gastrointestinal bleeding	2 (20)
7. Pseudocyst	2 (20)
8. Abscess	1 (10)
9. Infected necrosis	1 (10)

Table 6 Clinical and laboratory parameters

		Mild AP (n = 135)			Severe AP (n = 10)			
		Mean ± SE	n	Range	Mean ± SE	n	Range	P
		Patient criteria met			Patient criteria met			
1	BE ≤ −3 mEq	−2.6 ± 2.7	8	−6.1–1.9	−9.5 ± 2.5	9	−1.3—25	<0.05
	or shock	0	135		6	10		<0.01
2	PaO_2 ≤60 mmHg (room air)	NA			NA			
	or pulmonary insufficiency	0	135		4	10		<0.01
3	BUN ≥40 mg/dL	10.6 ± 0.45	135	2–26	28.9 ± 5.60	10	6–61	<0.01
	or oliguria (<0.5 mL/kg/h)	0	135		2	10		<0.01
4	LDH ≥2 × above upper limit	1.13 ± 0.05	119	0.32–3.56	2.12 ± 0.12	8	0.74–3.84	<0.01
5	Platelet count ≤1 × 10^5/mm^3	29.9 ± 1.0	135	2.3–67.7	12.4 ± 2.94	10	1.2–33.0	NS
6	Ca ≤7.5 mg/dL	9.53 ± 0.07	126	7.2–10.8	8.24 ± 0.21	10	7.4–9.6	NS
7	CRP ≥15 mg/dL	2.36 ± 0.29	135	0.1–15.5	12.7 ± 1.96	10	4.8–22.6	<0.01
8	Pediatric SIRS score ≥3	2	135	0–3	6	10	1–4	<0.01
9	Age <7 years and/or weight <23 kg	7.38 ± 0.03	135	0.8–17	6.40 ± 0.42	10	2–13	NS
		26.3 ± 1.3	135	7.3–64.1	20.9 ± 3.7	10	10.5–47.0	NS

AP, acute pancreatitis; BE, base excess; BUN, blood urea nitrogen; Ca, calcium; CRP, C-reactive protein; LDH, lactate dehydrogenase; PaO_2, partial pressure of arterial oxygen; SIRS, systemic inflammatory response syndrome.

Comparison of four scoring systems in predicting severity of AP

The means of the Ranson (1.15 ± 0.07 vs. 3.20 ± 0.49, $P \leq 0.01$), modified Glasgow (0.50 ± 0.06 vs. 2.76 ± 0.42, $P \leq 0.01$), DeBanto (1.41 ± 0.10 vs. 2.70 ± 0.47, $P \leq 0.01$), and pediatric JPN (0.82 ± 0.06 vs. 3.80 ± 0.49, $P \leq 0.01$) scores varied significantly between the mild and severe groups (Table 7). ROC curves were produced for each scoring system to compare the overall predictive value, without confining the analysis to any arbitrary cut-off (Fig. 1). On ROC analysis the AUC (mean ± SE) for the pediatric JPN score (0.980 ± 0.012) was superior to the Ranson (0.886 ± 0.056), modified Glasgow (0.935 ± 0.034), and DeBanto (0.754 ± 0.074) scoring systems (Fig. 1).

Using the cut-off of 3 points, pediatric JPN score had better sensitivity (80.0%) as compared to the Ranson (60.0%), modified Glasgow (50.0%), and DeBanto (60.0%) scores. The specificity was 96.3% for the pediatric JPN score, 93.6% for the Ranson score, 98.5% for the modified Glasgow score, and 86.0% for the DeBanto score. The positive predictive value of the modified Glasgow score (71.4%) was superior to the other scoring systems (pediatric JPN, 61.5%; Ranson, 42.8%; and DeBanto, 24.0%). The negative predictive value of the four systems was almost identical (pediatric JPN, 98.4%; Ranson, 96.7%; modified Glasgow, 96.4%; and DeBanto, 96.7%; Table 7).

Discussion

Alcohol and gallstones are the main etiologies of AP in adults.[11,12] The etiology in children, however, is often drugs, infection, trauma, or anatomic anomalies such as choledochal cyst and abnormal union of the pancreatobiliary junction.[1,7,13] Because the basic pathogenesis of AP does not greatly differ between adults and children, the current approach to pediatric AP relies on experience and knowledge gained from treating adult AP.[13] There are no data, however, on the mortality rate of severe AP in childhood. The mortality rate in Japanese adults decreased from 27% in 1996 to 8% in 2011 due to the development of new diagnostic and therapeutic methods.[14] Providing intensive care unit (ICU) management, specialized therapies such as continuous regional arterial infusion of protease inhibitor and antibiotics, enteral nutrition via nasojejunal feeding, and continuous hemodiafiltration have improved the outcome of severe AP by reducing infection and averting pancreatic surgery, even in children.[15–17] To reduce the mortality rate, it is important for patients with severe AP to be transferred to a medical facility with adequate monitoring and intensive care. Several scoring systems are used in adults to identify severe AP, but there is no universally accepted scoring system for predicting the severity of childhood AP.[1,6,7]

The JPN scoring system (2008 version), which is based on clinical signs, blood test data, and imaging, is widely used in Japan to determine treatment strategies.[5] The effectiveness of

Table 7 Prediction of severity of AP

System	Mild (mean ± SE)	Severe (mean ± SE)	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ranson score (maximum, 11 points)	1.15 ± 0.07	3.20 ± 0.49	84.8	60.0	93.6	42.8	96.7
Modified Glasgow score (maximum, 8 points)	0.50 ± 0.06	2.76 ± 0.42	95.2	50.0	98.5	71.4	96.4
DeBanto score (maximum, 8 points)	1.41 ± 0.10	2.70 ± 0.47	84.1	60.0	86.0	24.0	96.7
Pediatric JPN score (maximum, 9 points)	0.82 ± 0.06	3.80 ± 0.49	95.2	80.0	96.3	61.5	98.4

AP, acute pancreatitis; JPN, Ministry of Health, Labour and Welfare of Japan; NPV, negative predictive value; PPV, positive predictive value.

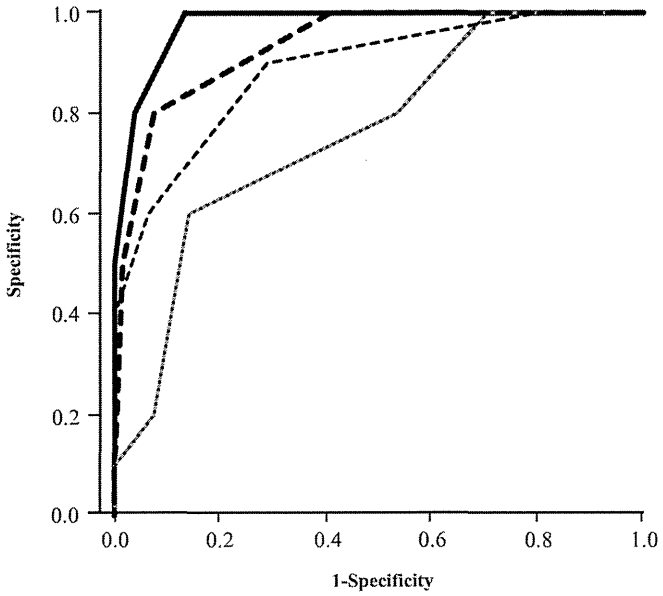

Fig. 1 Receiver operating characteristic curve of the discrimination of the (—) modified Ministry of Health, Labour and Welfare of Japan (pediatric JPN) score, (----) Ranson, (------) modified Glasgow, and (·····) DeBanto scores for predicting major complications in pediatric acute pancreatitis. The area under the curve (AUC) was greater for the pediatric JPN score (0.980) than for the Ranson (0.886), modified Glasgow (0.935), and DeBanto (0.754) prognostic scores.

CRP, which is a criterion in the JPN system, as a single marker for the prediction of severity has been reported,[18,19] but CRP is not a criterion in the Ranson, modified Glasgow or DeBanto scores. The cut-off for predicting severe outcome at CRP ≥15 mg/dL after 48 h from onset of AP was recommended at the Santorini consensus conference (1999),[20] in the World Congress of Gastroenterology guidelines (2002),[21] and in the UK guidelines (2005).[22] Unfortunately, the JPN scoring system lacks the predictive value needed to guide confident clinical decision-making in children. When the JPN system (2008 version) was used in the present patients, the specificity was 100% but the sensitivity was only 50%. In the present study, we modified only two parameters of the JPN scoring system to produce the pediatric JPN scoring system.

The AUC of the ROC curve provides a global assessment of test performance, without applying any arbitrary cut-off. In this analysis, the pediatric JPN score had the best overall discrimination. Using the cut-off of 3 points in each system, this new system had equal or better clinical accuracy (95.2%) as compared to the Ranson (84.8%), modified Glasgow (95.2%), and DeBanto systems (84.1%). The present study, however, has several limitations that are inherent to any retrospective study. Because mean platelets, serum Ca, BUN, and CRP did not reach the cut-off in the severe AP group, reconstruction of new suitable cut-offs for pediatric patients is needed to examine an increased number of severe AP cases. Additionally, the usefulness of the pediatric JPN score should be examined in Western children, because there are etiological differences in AP between Asian and Western children.[23,24]

The international consensus conference on pediatric sepsis and organ dysfunction was held in February 2002 in San Antonio, Texas, where the criteria for adult SIRS were modified for pediatric use, and the definitions of sepsis, severe sepsis, and septic shock for pediatric patients were revised.[9] It was determined that at least two of four criteria, one of which must be abnormal temperature or leukocyte count, are required to confirm SIRS in a pediatric patient. Age-specific normal values for vital signs and laboratory data were incorporated into the definitions of SIRS and sepsis in children. Because SIRS criteria were derived from the adult respiratory distress syndrome criteria, there was much discussion that this criteria contained higher sensitivity and lack of specificity. A literature review by Carvalho *et al.* cited one study reporting a 68% prevalence of SIRS in the pediatric ICU.[25] In adults, 68% of patients admitted to the ICU met at least two criteria for SIRS.[26] In Japan, the prevalence of SIRS reached 84% among all adult ICU patients.[27] These data are not fully comparable to the present ones due to differing patient age groups and cut-off criteria.

In many clinical settings, the very old and the very young are at the highest risk of poor outcome. Persons in these age groups often have lower physiological reserves than do otherwise healthy adults in the prime of life. In the present study, although there were no significant differences in age and weight among patients with mild or severe AP, the age (6.40 ± 0.42 years) and weight (20.9 ± 3.7 kg) in the severe group were similar to those in the first pediatric report on AP by DeBanto *et al.*[1] In contrast, in the second report by Lautz *et al.* patients younger than 7 years were more likely to have mild (29%) than severe AP (12.5%, $P < 0.05$).[7] Further examination is necessary to verify the cut-offs for age and weight in a validation group.

The CT severity index (CTSI) has proven to be very useful in adults.[5,28] Recently, Lautz *et al.* reported that CTSI was superior to a clinical scoring system for identifying children with AP at heightened risk for developing serious complications.[29] Although 42 of 145 patients received plain and/or contrast-enhanced CT, in the present study it was not possible to evaluate the performance of the CTSI because only three patients with severe AP received CT. Most patients received non-invasive abdominal echosonography instead of CT. Clinical trials assessing the usefulness of the CT scoring system in Japanese children and adolescents with AP are needed.

Conclusion

Pediatric JPN score, although not perfect, is sufficient for the prediction of outcome in the initial period in order to identify pediatric patients with severe AP. The use of this scoring system at admission may help to improve treatment outcome in pediatric patients.

Acknowledgments

This work was supported by grant no. 555 from the Pancreatic Research Foundation of Japan. The authors declare that they have no conflicts of interest.

References

1 DeBanto JR, Goday PS, Pedroso MR *et al.* Acute pancreatitis in children. *Am. J. Gastroenterol.* 2002; **97**: 1726–31.
2 Ranson JH, Rifkind KM, Roses DF, Fink SD, Eng K, Spencer FC. Prognostic signs and the role of operative management in acute pancreatitis. *Surg. Gynecol. Obstet.* 1974; **139**: 69–81.
3 Ranson JH. Etiological and prognostic factors in human acute pancreatitis: A review. *Am. J. Gastroenterol.* 1982; **77**: 633–8.
4 Blamey SL, Imrie CW, O'Neill J, Gilmour WH, Carter DC. Prognostic factors in acute pancreatitis. *Gut* 1984; **25**: 1340–46.
5 Takeda K, Yokoe M, Takada T *et al.* Assessment of severity of acute pancreatitis according to new prognostic factors and CT grading. *J. Hepatobiliary Pancreat. Sci.* 2010; **17**: 37–44.
6 Suzuki M, Fujii T, Takahiro K, Ohtsuka Y, Nagata S, Shimizu T. Scoring system for the severity of acute pancreatitis in children. *Pancreas* 2008; **37**: 222–3.
7 Lautz TB, Chin AC, Radhakrishnan J. Acute pancreatitis in children: Spectrum of disease and predictors of severity. *J. Pediatr. Surg.* 2011; **46**: 1144–9.
8 Ogawa M, Hirota M, Hayakawa T *et al.* Development and use of a new staging system for severe acute pancreatitis based on a nationwide survey in Japan. *Pancreas* 2002; **25**: 325–30.
9 Goldstein B, Giroir B, Randolph A. International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. *Pediatr. Crit. Care Med.* 2005; **6**: 2–8.
10 Bradley EL 3rd. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. *Arch. Surg.* 1993; **128**: 586–90.
11 Banks PA. Epidemiology, natural history, and predictors of disease outcome in acute and chronic pancreatitis. *Gastrointest. Endosc.* 2002; **56** (6 Suppl): S226–30.
12 Yadav D, Lowenfels AB. Trends in the epidemiology of the first attack of acute pancreatitis: A systematic review. *Pancreas* 2006; **33**: 323–30.
13 Nydegger A, Couper RT, Oliver MR. Childhood pancreatitis. *J. Gastroenterol. Hepatol.* 2006; **21**: 499–509.
14 Satoh K, Shimosegawa T, Masamune A *et al.* Nationwide epidemiological survey of acute pancreatitis in Japan. *Pancreas* 2011; **40**: 503–7.
15 Yasuda T, Ueda T, Takeyama Y *et al.* Treatment strategy against infection: Clinical outcome of continuous regional arterial infusion, enteral nutrition, and surgery in severe acute pancreatitis. *J. Gastroenterol.* 2007; **42**: 681–9.
16 Piascik M, Rydzewska G, Milewski J *et al.* The results of severe acute pancreatitis treatment with continuous regional arterial infusion of protease inhibitor and antibiotic: A randomized controlled study. *Pancreas* 2010; **39**: 863–7.
17 Fukushima H, Fukushima T, Suzuki R *et al.* Continuous regional arterial infusion effective for children with acute necrotizing pancreatitis even under neutropenia. *Pediatr. Int.* 2013; **55**: e11–13.
18 Viedma JA, Perez-Mateo M, Agullo J, Dominguez JE, Carballo F. Inflammatory response in the early prediction of severity in human acute pancreatitis. *Gut* 1994; **35**: 822–7.
19 Pezzilli R, Billi P, Miniero R *et al.* Serum interleukin-6, interleukin-8, and beta 2-microglobulin in early assessment of severity of acute pancreatitis. Comparison with serum C-reactive protein. *Dig. Dis. Sci.* 1995; **40**: 2341–8.
20 Dervenis C, Johnson CD, Bassi C *et al.* Diagnosis, objective assessment of severity, and management of acute pancreatitis. Santorini consensus conference. *Int. J. Pancreatol.* 1999; **25**: 195–210.
21 Toouli J, Brooke-Smith M, Bassi C *et al.* Guidelines for the management of acute pancreatitis. *J. Gastroenterol. Hepatol.* 2002; **17** (Suppl): S15–39.
22 UK Working Party on Acute Pancreatitis. UK guidelines for the management of acute pancreatitis. *Gut* 2005; **54** (Suppl 3): iii1–9.
23 Tomomasa T, Tabata M, Miyashita M, Itoh K, Kuroume T. Acute pancreatitis in Japanese and Western children: Etiologic comparisons. *J. Pediatr. Gastroenterol. Nutr.* 1994; **19**: 109–10.
24 Werlin SL, Kugathasan S, Frautschy BC. Pancreatitis in children. *J. Pediatr. Gastroenterol. Nutr.* 2003; **37**: 591–5.
25 Carvalho PR, Feldens L, Seitz EE, Rocha TS, Soledade MA, Trotta EA. Prevalence of systemic inflammatory syndromes at a tertiary pediatric intensive care unit. *J. Pediatr. (Rio J)* 2005; **81**: 143–8.
26 Rangel-Frausto MS, Pittet D, Costigan M, Hwang T, Davis CS, Wenzel RP. The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. *JAMA* 1995; **273**: 117–23.
27 Shibata K, Funada H. The epidemiology of SIRS. Sepsis in Japan. *Nihon Rinsho* 2004; **62**: 2184–8.
28 Balthazar EJ. Acute pancreatitis: Assessment of severity with clinical and CT evaluation. *Radiology* 2002; **223**: 603–13.
29 Lautz TB, Turkel G, Radhakrishnan J, Wyers M, Chin AC. Utility of the computed tomography severity index (Balthazar score) in children with acute pancreatitis. *J. Pediatr. Surg.* 2012; **47**: 1185–91.

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

Takao Togawa, MD[1], Tokio Sugiura, MD, PhD[1], Koichi Ito, MD, PhD[1], Takeshi Endo, MD, PhD[1], Kohei Aoyama, MD[1], Kei Ohashi, MD[1], Yutaka Negishi, MD[1], Toyoichiro Kudo, MD, PhD[2], Reiko Ito, MD, PhD[2], Atsuo Kikuchi, MD, PhD[3], Natsuko Arai-Ichinoi, MD, PhD[3], Shigeo Kure, MD, PhD[3], and Shinji Saitoh, MD, PhD[1]

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;■:■-■).*

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.

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		
BRIC	Benign recurrent intrahepatic cholestasis	NGS	Next-generation sequencing
CNV	Copy number variation	NICCD	Neonatal intrahepatic cholestasis caused by citrin deficiency
D.Bil	Direct bilirubin	NIIC	Neonatal/infantile intrahepatic cholestasis
DJS	Dubin-Johnson syndrome		
GGT	Gamma glutamyl transpeptidase	PFIC	Progressive familial intrahepatic cholestasis
HGVB	Human Genetic Variation Browser		
Ion PGM	Ion Torrent Personal Genome Machine	T.Bil	Total bilirubin

From the [1]Department of Pediatrics and Neonatology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [2]Department of Hepatology, National Medical Center for Children and Mothers, National Center for Child Health and Development, Tokyo, Japan; and [3]Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan

Supported by Grant-in-Aid for Young Scientists (26870489 [to T.S.]). The authors declare no conflicts of interest.

http://dx.doi.org/10.1016/j.jpeds.2016.01.006

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 as follows. The entry criteria included: (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). IR 4.0 could potentially call a copy number variation (CNV). The Human Gene Mutation Database professional in January 2015 (http://www.hgmd.org/, BioBase), Sorting Intolerant From Tolerant, Polymorphism Phenotyping, and Human Splicing Finder ver 3.0 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, URL: http://www.genome.med.kyoto-u.ac.jp/SnpDB) that was released in November 2013 and to the Exome Aggregation Consortium, Cambridge, Massachusetts (http://exac.broadinstitute.org; 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/). 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]

Preliminary Validation of the System on 5 Positive Control Cases

To evaluate the performance of our NGS-based molecular diagnosis system, we tested samples from 5 patients with NIIC genotyped by conventional Sanger sequencing. These

samples carried 9 disease-causing variants in total: 6 single-nucleotide variants, 1 small insertion, 1 small deletion, and 1 gross deletion (516 bp) in *JAG1*, *SLC25A13*, or *ABCB11*. The basic sequence data showed an average depth of coverage in the target regions of 600-fold and that 97.6% of the target regions had a coverage of more than 100-fold. We successfully identified all 9 variants, confirming the efficacy of our diagnostic system.

Comparison of the Clinical and Laboratory Findings among Subjects with the Molecular Diagnosis

The values of clinical and laboratory findings among subjects with a molecular genetic diagnosis were analyzed statistically.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6.05 for windows (GraphPad Software, Inc, La Jolla, California). Kruskal-Wallis nonparametric one-way ANOVA followed by Dunn multiple comparison test were performed, and a P value of $<.05$ was considered significant.

Results

One hundred nine subjects fulfilled our criteria and were enrolled in this study (**Figure 1**; available at www.jpeds.com). Of these 109 subjects, 31 (28%) were clinically diagnosed with genetic cholestasis (**Table II**). For all subjects, the median age of onset of symptoms associated with cholestasis was 1 month, and the median D.Bil value was 4.1 mg/dL. The median age of the subjects when we performed the NGS screening was 5 months (2-15 months, IQR).

Summary of Ion PGM Sequencing

The median number of total sequenced bases per subject, of mapped reads, and of mean read length were 61.9 mega bases (42.7-76.9, IQR), 414 kilo reads (294-525, IQR), and 148 bases (139-152, IQR), respectively. The average depth of coverage in the target region was 672-fold (447-849, IQR); 97.0% of the target regions had more than 100-fold coverage.

Molecular Genetic Diagnosis

Our sequencing and bioinformatic analysis indicated that 28 (26%) subjects received a molecular genetic diagnosis of pathogenic or likely pathogenic variants of *JAG1*, *NOTCH2*, *ABCC2*, *SLC25A13*, *ATP8B1*, or *ABCB11*. The molecular genetic diagnosis in 22 (79%) of the 28 subjects was consistent with the clinical diagnosis, as these subjects had been categorized as "genetic cholestasis." For the remaining 6 subjects who had been clinically categorized as "unknown with complications" (2 subjects) or "unknown without complications" (4 subjects), the molecular genetic diagnosis was as follows: 1 with ALGS, 3 with neonatal DJS, 1 with NICCD, and 1 with PFIC2/BRIC2. **Table III** (available at www.jpeds.com) shows the characteristics of the variants in the 28 subjects with a molecular genetic diagnosis. We

Table II. Baseline demographic and clinical characteristics*

Clinical diagnosis	No.	Sex (male)	Age (mo) at: Onset	Age (mo) at: NGS†	GA (w)	BW (g)	T.Bil (mg/dL)	D.Bil (mg/dL)	AST (IU/L)	ALT (IU/L)	GGT (IU/L)
Genetic cholestasis	31	13									
ALGS	13	5	1 (0-2)	4 (2-15)	38 (37-39)	2584 (2306-2790)	10.7 (6.0-14.2)	7.0 (4.4-9.0)	231 (112-299)	159 (70-293)	290 (106-1023)
Neonatal DJS	2	2	0 (0-0)	1.5 (1-2)	39 (39-39)	3135 (2774-3496)	16.8 (10.2-23.4)	9.8 (6.2-13.4)	32 (19-44)	19 (12-25)	148 (97-199)
NICCD	4	3	4 (4-6)	6 (4-12)	40 (39-41)	2768 (2546-3148)	5.2 (2.5-10)	2.3 (1.4-3.3)	124 (106-194)	58 (29-97)	192 (104-273)
PFIC/BRIC	12	3	2 (1-2)	10 (4-20)	38 (37-39)	2863 (2585-3255)	10.8 (3.4-15.1)	6.4 (2.2-11.4)	109 (45-233)	62 (30-153)	48 (16-67)
Unknown, complication (+)	46	25									
Perinatal abnormalities	31	17	1 (0-3)	5 (2-8)	34 (28-37)	1760 (774-2360)	6.2 (5.1-7.7)	4.1 (2.9-5.4)	125 (50-206)	54 (21-122)	110 (59-207)
Infections	7	3	2 (1-2)	3 (1-5)	39 (38-40)	3252 (2995-3380)	5.2 (3.5-10.8)	2.6 (2.4-7.1)	149 (42-836)	130 (13-517)	84 (59-204)
Metabolic acidosis	3	2	1 (1-9)	2 (1-11)	39 (37-40)	2675 (1992-2824)	7.3 (6.8-9.5)	4.5 (2.1-4.8)	2164 (143-4190)	874 (56-1673)	222 (151-317)
Abnormal hormone values	3	2	1 (0-2)	17 (2-33)	39 (36-40)	2850 (2190-3120)	10.8 (2.3-11.4)	4.2 (1.0-8.7)	137 (56-263)	43 (26-185)	100 (89-237)
Hemochromatosis	1	1	0	13	37	2122	12.7	4.6	166	31	26
Milk allergy	1	0	0	1	39	2808	14.2	3.1	50	12	1247
Unknown, complication (−)	32	16	1 (0-3)	10 (3-25)	39 (38-40)	2920 (2734-3159)	6.8 (5.3-10.9)	3.7 (2.3-7.2)	75 (48-140)	49 (30-100)	81 (45-119)
Total	109	54	1 (0-3)	5 (2-15)	38 (37-39)	2720 (2253-3045)	7.0 (5.2-10.9)	4.1 (2.6-7.0)	113 (53-213)	59 (30-151)	90 (58-215)

BW, birth weight; *GA*, gestational age; *No.*, number.
*Data are median values (IQR).
†The median age of the subjects when we performed the NGS screening.

confirmed all the variants shown in **Table III** by using conventional Sanger sequencing or MLPA analysis.

Eleven of the 13 clinically diagnosed ALGS patients had genetic mutations in either *JAG1* or *NOTCH2*; we identified 10 heterozygous pathogenic or likely pathogenic variants in *JAG1* (NCU01-10) and 1 likely pathogenic variant in *NOTCH2* (NCU11). We also identified 1 subject (NCU23) with a *JAG1* missense mutation in the "unknown with complications" group. This subject was a preterm infant with infantile cholestasis and ALGS-like facial features but no other symptoms. Thus, 12 subjects in total were defined as having ALGS by molecular genetic diagnosis. Regarding variant type in *JAG1*, we identified 1 subject (NCU10) with a deletion encompassing an entire exon, which was called by IR 4.0 as CNV 1 in the region of chr20: 10 619 976-10 654 275. We confirmed the deletion in *JAG1* using the SALSA MLPA probemix P184-C2 *JAG1* (MRC-Holland bv; Amsterdam, the Q3 Netherlands; data not shown).

Two subjects (NCU12, 13) who had been clinically diagnosed with neonatal DJS due to a black liver in macroscopic findings had compound heterozygous pathogenic or likely pathogenic variants in *ABCC2*. Surprisingly, 3 subjects (NCU25-27) who had been categorized under the group "unknown without complications" had pathogenic variants in a compound heterozygous state. These 3 subjects showed a normal value of serum AST/ALT, and their D.Bil decreased spontaneously; however, they did not have a black liver, as determined by macroscopic or microscopic study following experimental laparotomy. Thus, 5 subjects were confirmed with neonatal DJS by molecular genetic diagnosis.

All 4 subjects (NCU14-17) who had been diagnosed clinically with NICCD were confirmed by molecular genetic diagnosis as having compound heterozygous pathogenic variants in *SLC25A13*. We also identified 1 subject (NCU28) with compound heterozygous pathogenic variants in *SLC25A13* from the "unknown without complications" group. Subject NCU28 presented clinically with prolonged jaundice and failure to thrive but with a normal newborn screening test and a normal serum amino acid profile.

Five of the 12 subjects that were diagnosed clinically with PFIC/BRIC were shown by molecular genetic diagnosis to have compound heterozygous pathogenic or likely pathogenic variants; 2 (NCU18, 19) in *ATP8B1* and 3 (NCU20-22) in *ABCB11*, which molecularly confirmed these subjects to have PFIC1/BRIC1 and PFIC2/BRIC2, respectively. One subject (NCU24), who had been categorized as "unknown with complications," was molecularly diagnosed with compound heterozygous likely pathogenic variants in *ABCB11*.

No molecular genetic diagnosis was confirmed in *ABCB4*, *TJP2*, *HSD3B7*, *AKR1D1*, *CYP7B1*, *VPS33B*, *BAAT*, *EPHX1*, *SLC10A1*, *ABCB1*, *SLCO1A2*, or *SLC4A2*. However, we found 12 subjects with a pathogenic or a likely pathogenic variant on a single allele in an AR disease gene: 5 subjects in *ABCC2*, 1 in *SLC25A13*, 5 in *ABCB11*, and 1 in *ABCB4*. Therefore, these subjects did not receive a definitive molecular genetic diagnosis.

Comparison of Clinical and Laboratory Findings between the 28 Subjects with Those of the Molecular Genetic Diagnosis

Statistical significance was detected for age of onset, gestational age, birth weight, D.Bil, AST, ALT, and GGT between Q4 subjects of ALGS, neonatal DJS, NICCD, and PFIC/BRIC (**Figure 2**). The age of onset of neonatal DJS was [F2] significantly earlier than that of NICCD (0 months vs 4 months), and the D.Bil level also was significantly greater than that of NICCD (11 mg/dL vs 2.6 mg/dL) (**Figure 2**, A and B). Birth weight in ALGS was significantly smaller than that in neonatal DJS (2538 g vs 2936 g), and, on the other hand, AST/ALT levels in neonatal DJS were significantly lower than those in ALGS (25/16 IU/L vs 265/196 IU/L) (**Figure 2**, C-E). As might be predicted, the GGT level in PFIC/BRIC was significantly lower than that in ALGS (17 IU/L vs 513 IU/L) (**Figure 2**, F). Unexpectedly, the gestational age in PFIC/BRIC was significantly earlier than that in NICCD (37.5 weeks vs 40 weeks). No significant differences were found, however, in the T.Bil, total bile acids, or total cholesterol levels between these groups.

Discussion

In this study, we analyzed 109 patients with NIIC, and a molecular genetic diagnosis was made for 26% of them (28/109). We categorized the subjects into 3 groups: "genetic cholestasis" including ALGS, neonatal DJS, NICCD, and PFIC/BRIC (31 subjects); "unknown with complications" (32 subjects), and "unknown without complications" (46 subjects). The positive molecular genetic diagnosis rate in the "genetic cholestasis" group was 71% (22/31). It is noteworthy that the results of all the molecular diagnoses were consistent with those of clinical diagnosis in this group. Thus, precise clinical evaluation would be warranted to predict underlying genetic diagnosis. Nevertheless, a patient with clinical features of ALGS was reported to have compound heterozygous variants of *ATP8B1* without pathogenic variants of *JAG1* or *NOTCH2*,[21] indicating potential genetic heterogeneity in NIIC. Forty-six subjects were clinically categorized as "unknown with complications," and a molecular genetic diagnosis was made in 2 subjects (4.3%). The low positive rate could be explained by the existence of other complications that may have caused the NIIC, such as prematurity, infection, and metabolic or hormonal system abnormality.[3,22] Thirty-two subjects were categorized clinically under "unknown without complications" and a molecular genetic diagnosis was made in 4 subjects (12.5%), which supported the aforementioned finding. Taken together, 6 subjects (7.7%) of unknown etiology were successfully categorized by the molecular genetic diagnosis; 1 with ALGS, 3 with neonatal DJS, 1 with NICCD, and 1 with PFIC2/BRIC2. Molecular genetic diagnosis is undoubtedly important to confirm the clinical diagnosis and to allow the provision of appropriate genetic counseling.[8,14,20,23] Therefore, targeted NGS as reported in this study would be a powerful and practical

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

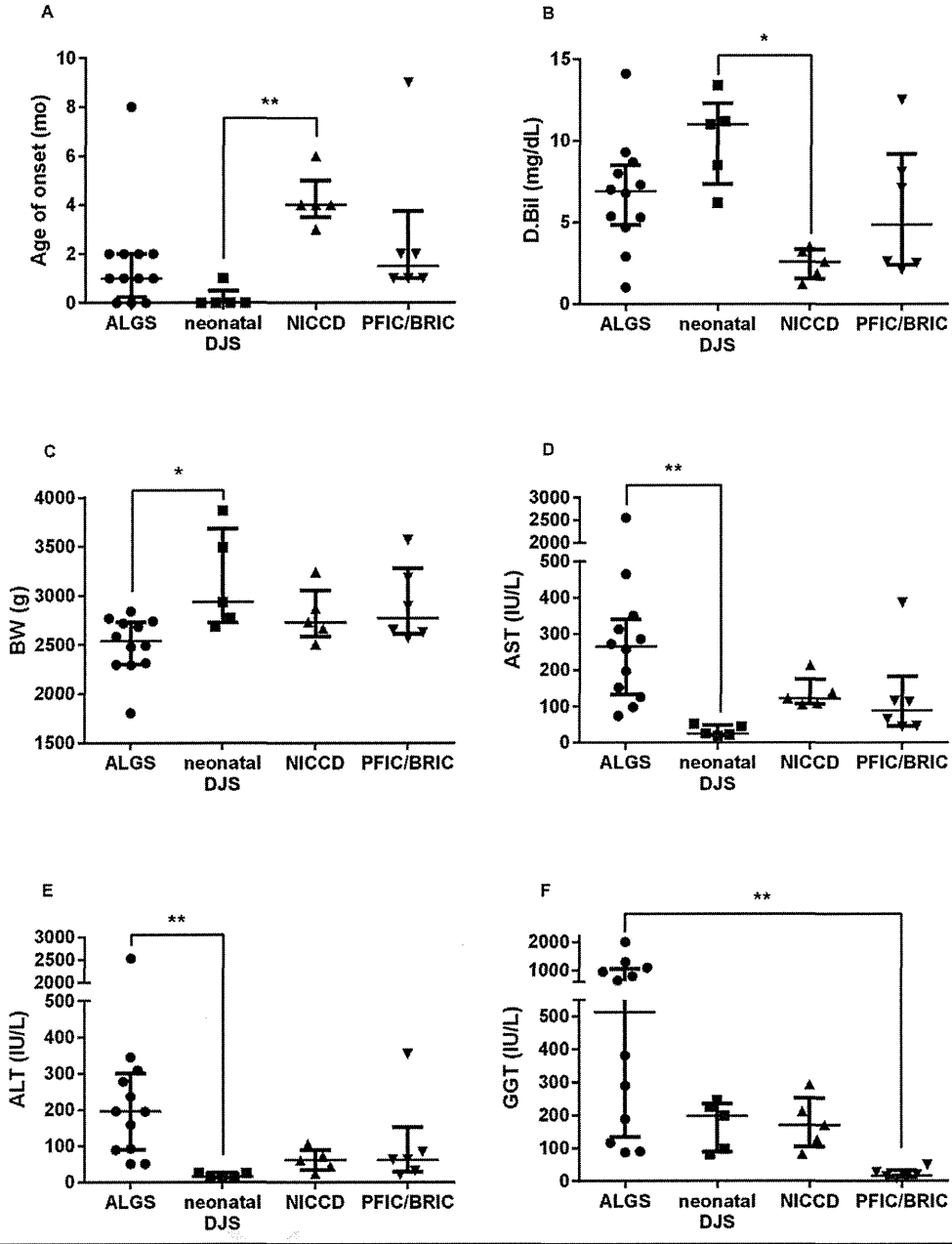

Figure 2. A, The age of onset; **B,** serum D.Bil; **C,** birth weight (BW); **D,** AST; **E,** ALT; and **F,** GGT were compared for the indicated groups of subjects. Kruskal-Wallis nonparametric one-way ANOVA followed by Dunn multiple comparison test were performed. $^{*}P < .05$; $^{**}P < .01$; error bars, IQR.

solution for the diagnosis of NIIC, especially for patients with atypical clinical presentation.

In our assay, we detected 1 whole exon deletion, 6 small heterozygous deletions, and 1 small heterozygous insertion using 2 different variant calling algorithms other than single-nucleotide variants (**Table III**). Notably, we identified whole exon deletion of *JAG1* with IR 4.0, in which the CNV calling is based on sequenced read depth. In a previous report of molecular genetic diagnosis for patients with NIIC, Liu et al[23,24] developed a hybridization-based resequencing chip, the *Jaundice Chip*, which includes 5 disease-causing genes; *SERPINA1*, *JAG1*, *ATP8B1*, *ABCB11*, and *ABCB4*. Single-nucleotide variants were detected with high accuracy by this chip. However, the authors stated that heterozygous deletions or insertions might not be detected by this chip because the strand with normal sequences would produce a normal readout.

According to the Human Gene Mutation Database professional, more than 450 *JAG1* disease-causing variants have been registered, and approximately 50% of these have been

classified as gross deletions or small indels. Thus, the approach to a CNV calling with a potentially predictable bioinformatic tool should be performed in targeted NGS for patients with NIIC. Recently, Herbst et al[25] performed NGS of 6 children with infantile cholestasis for analysis of 93 genes with partially overlapping phenotypes associated with inherited cholestatic diseases. Using the Illumina MiSeq system, they made a molecular genetic diagnosis in 4 of the 6 patients and identified 6 novel variants in *PKHD1*, *ABCB11*, and *NPC1*, thus supporting the alternative NGS approach. In cases in which no variant or only a single allelic variant in a candidate AR gene are identified with targeted NGS, a further genetic investigation, such as whole-genome sequencing or an analysis of highly conserved promoter sequences in the noncoding region, might be applied.

Previous reports suggest that a heterozygous variant in *ATP8B1*, *ABCB11*, or *ABCB4* may play a role in the susceptibility to infantile intrahepatic cholestasis.[26-28] We found 5 subjects with a single heterozygous variant in *ABCB11*, including 4 missense mutations and 1 small insertion at the essential splice site, with no other candidate pathogenic variants. All of them showed a low GGT value in serum, and 2 subjects presented complete remission of cholestasis, suggesting a clinical diagnosis of PFIC/BRIC. Therefore, it is plausible that another mutation remained unidentified in this gene, or that a single heterozygous alteration might predispose to cholestasis. This issue remains to be solved in future studies.

We identified 5 neonatal DJS (including 2 siblings) subjects by molecular genetic analysis. In particular, 2 of these 5 subjects were identified as early as 2 months of age. DJS is diagnosed rarely in the infantile period, and only 5 neonatal DJS cases have undergone molecular genetic analysis.[9-11] Therefore, our two 2-month-old cases are the earliest cases to be diagnosed by molecular genetic analysis.[9-11] Six pathogenic or likely pathogenic variants in *ABCC2*, p.R100Ter, c.1815+2T>A, c.1967+2T>C, p.R768W, c.2439+2T>C, and p.K961R, were identified in our neonatal DJS (**Table III**). The p.R768W occurred with a high frequency, with 3 of 10 affected alleles in our genetically confirmed subjects and the MAF was registered as 0.252% in HGVB. We therefore suspected the presence of high-frequency pathogenic variants in *ABCC2* of neonatal DJS cases in the Japanese population such as in *SLC25A13* of NICCD.[12,29] The total MAF of these 6 variants in Japanese and East Asian or non-East Asian populations was calculated by HGVB and Exome Aggregation Consortium as 0.935%, 0.104%, and 0.00888%, respectively (**Table III**). The cumulative carrier rates of the 6 variants were predicted as approximately 1/54, 1/483, and 1/5634 on the basis of the Hardy-Weinberg principle. Thus, the estimated incidence of the affected patients with the 6 variants was calculated as approximately 1/12 000, 1/930 000, and 1/130 000 000. We speculated that cases of neonatal DJS would be more frequent in the Japanese population than in East Asian and non-East Asian populations, or that the constituent of causative pathogenic variants of neonatal DJS would be diverse between the ethnic groups.

We performed statistical analysis of the values of clinical findings for 28 subjects with a molecular genetic diagnosis. In our study, the characteristic clinical/laboratory findings of neonatal DJS showed the greatest serum value of D.Bil, and the earliest age of onset (11 mg/dL and 0 months, respectively). In ALGS, the median age of onset, AST/ALT, and GGT were 1 month, 265/196 IU/L, and 513 IU/L, respectively. Also, the GGT value in PFIC/BRIC clearly showed a low level (17 IU/L, median). These findings might help to make a differential diagnosis among conjugated hyperbilirubinemia in early infancy.

Our NIIC panel could be used in future application in clinical settings to detect disease-causing pathogenic variants as early as possible, to decrease the need of invasive procedures, to give disease-specific treatment, and to provide genetic counseling for the families. All of our patients with neonatal DJS underwent exploratory laparotomy or laparoscopy to exclude biliary atresia within 2 months of age, and 3 of them did not display a black liver. Early introduction of NGS-based molecular diagnosis would have made a proper diagnosis before the surgical intervention in these patients. Now, we have several alternatives to NGS-based molecular genetic diagnosis. Whole-exome sequencing can identify causative genetic alterations; however, whole-exome sequencing is still expensive and interpretation of results is time consuming. Small panel-based approaches are cheap, fast, and easy to interpret.[30] They also can identify CNVs, not detected by Sanger sequencing, as shown in this study. Therefore, small panels, including ours, are a practical approach for patients with NIIC, especially in early infancy, to avoid unnecessary surgical interventions.

This study presents some limitations. First, we could not access a large number of controls samples to measure the accuracy and analytical sensitivity of our assay. The accuracy and sensitivity of Ion PGM based sequencing has been reported to be compatible with other methods,[30] but we only tested our panel using 5 positive control patients with 9 disease-causing variants in total. Therefore, some mutations may not be detectable with our method. Second, our diagnostic gene panel was customized for Japanese patients with NIIC. Some cholestatic genes that are important for patients in western countries are not included, such as cystic fibrosis and alpha-antitrypsin deficiency, etc and NICCD is common in Japanese population.[29,31] An expanded panel encompassing more relevant genes could be used for the further investigations including diverse ethnic population.

In conclusion, we successfully achieved molecular genetic diagnosis in 28 (26%) of 109 Japanese patients with NIIC using targeted NGS and bioinformatics. Targeted NGS is a powerful tool that is feasible for the diagnosis of NIIC and should be introduced for early diagnosis of NIIC of unknown etiology as well as for the confirmation of clinically diagnosed genetic cholestasis syndromes. ■

We thank Drs Kenji Goto and Kohachiro Sugiyama (both deceased) for their help.

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

Submitted for publication Oct 10, 2015; last revision received Dec 21, 2015; accepted Jan 5, 2016.

Reprint requests: Dr Tokio Sugiura, MD, PhD, Department of Pediatrics and Neonatology, Nagoya City University Graduate School of Medical Sciences, Kawasumi-1, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. E-mail: tokio@med.nagoya-cu.ac.jp

References

1. Balistreri WF, Bezerra JA, Jansen P, Karpen SJ, Shneider BL, Suchy FJ. Intrahepatic cholestasis: summary of an American Association for the Study of Liver Diseases single-topic conference. Hepatology 2005;42: 222-35.
2. Balistreri WF, Bezerra JA. Whatever happened to "neonatal hepatitis"? Clin Liver Dis 2006;10:27-53.
3. Feldman AG, Sokol RJ. Neonatal Cholestasis. Neoreviews 2013;14.
4. Sambrotta M, Strautnieks S, Papouli E, Rushton P, Clark BE, Parry DA, et al. Mutations in TJP2 cause progressive cholestatic liver disease. Nat Genet 2014;46:326-8.
5. Koshimizu E, Miyatake S, Okamoto N, Nakashima M, Tsurusaki Y, Miyake N, et al. Performance comparison of bench-top next generation sequencers using microdroplet PCR-based enrichment for targeted sequencing in patients with autism spectrum disorder. PLoS One 2013;8:e74167.
6. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, et al. Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 2012;30:434-9.
7. Moyer V, Freese DK, Whitington PF, Olson AD, Brewer F, Colletti RB, et al. Guideline for the evaluation of cholestatic jaundice in infants: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr 2004;39: 115-28.
8. Turnpenny PD, Ellard S. Alagille syndrome: pathogenesis, diagnosis and management. Eur J Hum Genet 2012;20:251-7.
9. Lee JH, Chen HL, Chen HL, Ni YH, Hsu HY, Chang MH. Neonatal Dubin-Johnson syndrome: long-term follow-up and MRP2 mutations study. Pediatr Res 2006;59:584-9.
10. Pacifico L, Carducci C, Poggiogalle E, Caravona F, Antonozzi I, Chiesa C, et al. Mutational analysis of ABCC2 gene in two siblings with neonatal-onset Dubin Johnson syndrome. Clin Genet 2010;78:598-600.
11. Okada H, Kusaka T, Fuke N, Kunikata J, Kondo S, Iwase T, et al. Neonatal Dubin-Johnson syndrome: Novel compound heterozygous mutation in the ABCC2 gene. Pediatr Int 2014;56:e62-4.
12. Ohura T, Kobayashi K, Tazawa Y, Abukawa D, Sakamoto O, Tsuchiya S, et al. Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). J Inherit Metab Dis 2007; 30:139-44.
13. Pawlikowska L, Strautnieks S, Jankowska I, Czubkowski P, Emerick K, Antoniou A, et al. Differences in presentation and progression between severe FIC1 and BSEP deficiencies. J Hepatol 2010;53:170-8.
14. van Mil SW, van der Woerd WL, van der Brugge G, Sturm E, Jansen PL, Bull LN, et al. Benign recurrent intrahepatic cholestasis type 2 is caused by mutations in ABCB11. Gastroenterology 2004;127:379-84.
15. Davit-Spraul A, Gonzales E, Baussan C, Jacquemin E. Progressive familial intrahepatic cholestasis. Orphanet J Rare Dis 2009;4:1.
16. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res 2003;31:3812-4.
17. Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. Nucleic Acids Res 2002;30:3894-900.
18. Desmet F-O, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 2009;37:e67.
19. 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.
20. Jurkiewicz D, Gliwicz D, Ciara E, Gerfen J, Pelc M, Piekutowska-Abramczuk D, et al. Spectrum of JAG1 gene mutations in Polish patients with Alagille syndrome. J Appl Genet 2014;55:329-36.
21. Grochowski CM, Rajagopalan R, Falsey AM, Loomes KM, Piccoli DA, Krantz ID, et al. Exome sequencing reveals compound heterozygous mutations in ATP8B1 in a JAG1/NOTCH2 mutation-negative patient with clinically diagnosed Alagille syndrome. Am J Med Genet A 2015;167A: 891-3.
22. Jancelewicz T, Barmherzig R, Chung CT, Ling SC, Kamath BM, Ng VL, et al. A screening algorithm for the efficient exclusion of biliary atresia in infants with cholestatic jaundice. J Pediatr Surg 2015;50:363-70.
23. Matte U, Mourya R, Miethke A, Liu C, Kauffmann G, Moyer K, et al. Analysis of gene mutations in children with cholestasis of undefined etiology. J Pediatr Gastroenterol Nutr 2010;51:488-93.
24. 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.
25. Herbst SM, Schirmer S, Posovszky C, Jochum F, Rodl T, Schroeder JA, et al. Taking the next step forward - Diagnosing inherited infantile cholestatic disorders with next generation sequencing. Mol Cell Probes 2015; 29:291-8.
26. Jacquemin E, Malan V, Rio M, Davit-Spraul A, Cohen J, Landrieu P, et al. Heterozygous FIC1 deficiency: a new genetic predisposition to transient neonatal cholestasis. J Pediatr Gastroenterol Nutr 2010;50:447-9.
27. Hermeziu B, Sanlaville D, Girard M, Leonard C, Lyonnet S, Jacquemin E. Heterozygous bile salt export pump deficiency: a possible genetic predisposition to transient neonatal cholestasis. J Pediatr Gastroenterol Nutr 2006;42:114-6.
28. Davit-Spraul A, Gonzales E, Baussan C, Jacquemin E. The spectrum of liver diseases related to ABCB4 gene mutations: pathophysiology and clinical aspects. Semin Liver Dis 2010;30:134-46.
29. Tabata A, Sheng JS, Ushikai M, Song YZ, Gao HZ, Lu YB, et al. Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency. J Hum Genet 2008;53:534-45.
30. Li X, Buckton AJ, Wilkinson SL, John S, Walsh R, Novotny T, et al. Towards clinical molecular diagnosis of inherited cardiac conditions: a comparison of bench-top genome DNA sequencers. PLoS One 2013;8: e67744.
31. Yamashiro Y, Shimizu T, Oguchi S, Shioya T, Nagata S, Ohtsuka Y. The estimated incidence of cystic fibrosis in Japan. J Pediatr Gastroenterol Nutr 1997;24:544-7.

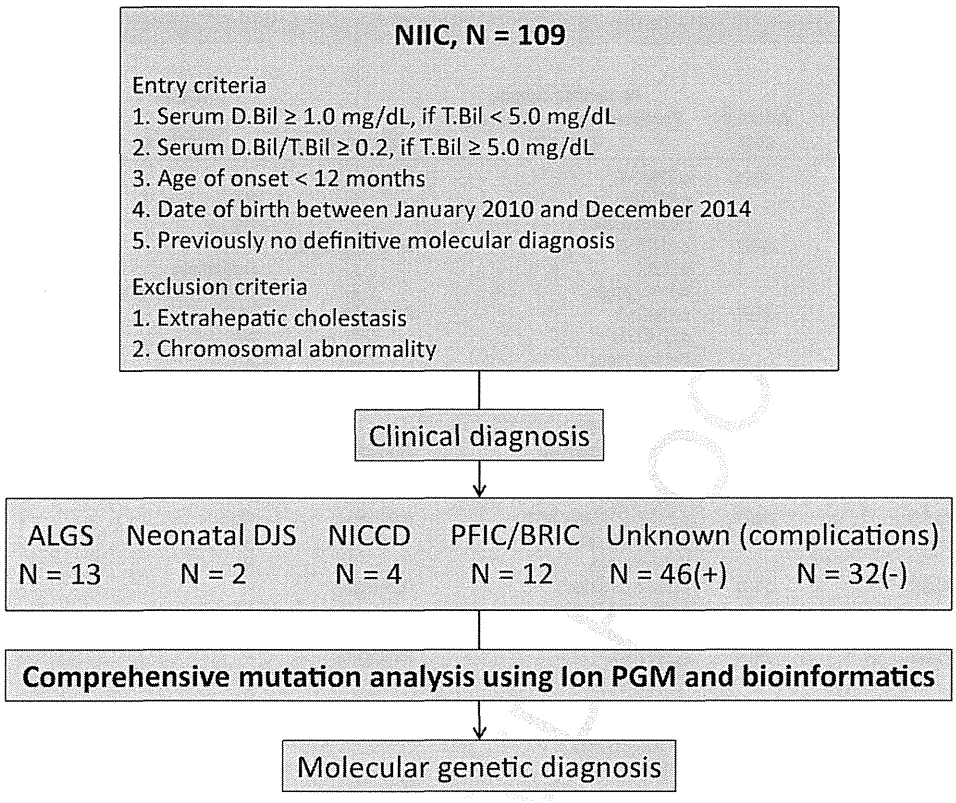

Figure 1. Flow diagram of the study and study subjects.

Table I. The panel of 18 targeted genes

Gene name	Disorder/function	No. exons	No. amplicons	Total no. targeted bases	% of coverage of target sequence
JAG1	ALGS	26	35	3657	99.9
NOTCH2	ALGS	35	63	7602	97.9
ABCC2	DJS	32	44	4638	99.7
SLC25A13	NICCD	19	28	2116	99.1
ATP8B1	PFIC1/BRIC1	27	45	3756	98.1
ABCB11	PFIC2/BRIC2	27	43	3966	99.0
ABCB4	PFIC3	28	44	4068	97.6
TJP2	PFIC4, FHCA	27	38	4158	95.6
HSD3B7	BAS	7	9	1170	100
AKR1D1	BAS	10	12	999	99.6
CYP7B1	BAS	6	17	1521	92.6
VPS33B	ARCS1	23	23	1854	100
BAAT	FHCA	3	10	1257	100
EPHX1	FHCA	8	15	1368	100
SLC10A1	NTCP	5	10	1050	98.7
ABCB1	MDR1	27	46	3843	99.2
SLC4A2	AE2	24	38	3759	98.6
SLCO1A2	OATP-1	14	26	2013	96.9
	Total	348	546	52 795	98.5

AE2, anion exchange protein 2; *ARCS1*, arthrogryposis, renal dysfunction and cholestasis syndrome-1; *BAS*, bile acid synthetic defect; *FHCA*, familial hypercholanemia; *MDR1*, multidrug resistance protein 1; *No.*, number; *NTCP*, Na(+)/taurocholate transport protein; *OATP-1*, organic anion transporting polypeptide 1.

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

Table III. Characteristics of the variants in the 28 subjects with a molecular genetic diagnosis

Subjects	Sex	Clinical diagnosis	Affected gene	Nucleotide change Predicted amino acid change Zygosity	Classification*	HGMD SIFT PolyPhen	MAF HGVB ExAC (ESA) ExAC (Non-ESA)	Parental origin
NCU01	F	ALGS	*JAG1*	c.238A>T	Pathogenic	-	-	De novo
				p.K80Ter		NA	-	
				Heterozygous		NA	-	
NCU02	M	ALGS	*JAG1*	c.359T>C	Likely Pathogenic	-	-	ND
				p.I120T		Deleterious	-	
				Heterozygous		possibly damaging	-	
NCU03	F	ALGS	*JAG1*	c.439C>T	Pathogenic	DM	-	Maternal
				p.Q147Ter		NA	-	
				Heterozygous		NA	-	
NCU04	M	ALGS	*JAG1*	c.551G>A	Pathogenic	DM	-	ND
				p.Arg184His		Deleterious	-	
				Heterozygous		probably damaging	-	
NCU05	F	ALGS	*JAG1*	c.785_789delGTGAT	Pathogenic	DM	-	De novo
				p.Cys262fsTer1		NA	-	
				Heterozygous		NA	-	
NCU06	F	ALGS	*JAG1*	c.2122_2125delCAGT	Pathogenic	DM	-	ND
				p.Q708fsTer34		NA	-	
				Heterozygous		NA	-	
NCU07	M	ALGS	*JAG1*	c.2767_2768insG	Pathogenic	-	-	De novo
				p.Asp923fsTer29		NA	-	
				Heterozygous		NA	-	
NCU08	F	ALGS	*JAG1*	c.2927delC	Pathogenic	-	-	De novo
				p.T976fsTer8		NA	-	
				Heterozygous		NA	-	
NCU09	F	ALGS	*JAG1*	c.3007_3010delGAGC	Pathogenic	-	-	De novo
				p.E1003fsTer32		NA	-	
				Heterozygous		NA	-	
NCU10	F	ALGS	*JAG1*	Whole exons deletion (Chromosome 20: 10 619 976-10 654 275)	Pathogenic	-	-	De novo
						NA	-	
						NA	-	
NCU11	M	ALGS	*NOTCH2*	c.5758G>C	Likely pathogenic	-	-	Maternal
				p.A1920P		Deleterious	-	
				Heterozygous		probably damaging	-	
NCU12	M	Neonatal DJS	*ABCC2*	c.1815+2T>A	Pathogenic	DM	-	Maternal
				Splice-site disruption		NA	1.16e-04	
				Heterozygous		NA	-	
			ABCC2	c.2302C>T	Pathogenic	DM	2.52e-03	Paternal
				p.R768W		Deleterious	3.47e-04	
				Heterozygous		probably damaging	5.33e-05	
NCU13	M	Neonatal DJS	*ABCC2*	c.2302C>T	Pathogenic	DM	2.52e-03	Paternal
				p.R768W		Deleterious	3.47e-04	
				Heterozygous		probably damaging	5.33e-05	
			ABCC2	c.2882A>G	Likely pathogenic	-	2.74e-03	Maternal
				Splice-site disruption (p.K961R†)		Tolerated	3.50e-04	
				Heterozygous		benign	-	
NCU14	M	NICCD	*SLC25A13*	c.615+1G>C	Pathogenic	DM	-	Maternal
				Splice-site disruption		NA	-	
				Heterozygous		NA	-	
			SLC25A13	c.1592G>A	Pathogenic	DM	1.67e-03	Paternal
				p.G531Asp		Deleterious	-	
				Heterozygous		probably damaging	-	
NCU15	M	NICCD	*SLC25A13*	c.674C>A	Pathogenic	DM	1.67e-03	Maternal
				p.S225Ter		NA	1.16e-04	
				Heterozygous		NA	-	
			SLC25A13	c.852_855delTATG	Pathogenic	DM	2.06e-03	Paternal
				p.R284fsTer3		NA	3.71e-03	
				Heterozygous		NA	-	
NCU16	M	NICCD	*SLC25A13*	c.852_855delTATG	Pathogenic	DM	2.06e-03	ND
				p.R284fsTer3		NA	3.71e-03	
				Heterozygous		NA	-	
			SLC25A13	c.1078C>T	Pathogenic	DM	-	ND
				p.R360Ter		NA	1.16e-04	
				Heterozygous		NA	3.55e-05	

(*continued*)

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

Table III. Continued

Subjects	Sex	Clinical diagnosis	Affected gene	Nucleotide change Predicted amino acid change Zygosity	Classification*	HGMD SIFT PolyPhen	MAF HGVB ExAC (ESA) ExAC (Non-ESA)	Parental origin
NCU17	F	NICCD	*SLC25A13*	c.852_855delTATG p.R284fsTer3 Heterozygous	Pathogenic	DM NA NA	2.06e-03 3.71e-03 -	Paternal
			SLC25A13	c.1177+1G>A Splice site disruption Heterozygous	Pathogenic	DM NA NA	3.18e-03 1.04e-03 -	Maternal
NCU18	F	PFIC/BRIC	*ATP8B1*	c.916T>C p.C306R Heterozygous	Pathogenic	DM Deleterious probably damaging	- - -	Maternal
			ATP8B1	c.2854C>T p.R952Ter Heterozygous	Pathogenic	DM NA NA	- - 8.87e-06	Paternal
NCU19	F	PFIC/BRIC	*ATP8B1*	c.922G>A p.G308S Heterozygous	Likely Pathogenic	- Deleterious probably damaging	- - -	Paternal
			ATP8B1	c.3579_3589delACGGCAGCAGG p.R1193fsTer39 Heterozygous	Pathogenic	- NA NA	- - -	Maternal
NCU20	F	PFIC/BRIC	*ABCB11*	c.386G>A p.C129Y Heterozygous	Likely Pathogenic	- Tolerated possibly damaging	- - -	Maternal
			ABCB11	c.1460G>A p.R487H Heterozygous	Pathogenic	DM Tolerated possibly damaging	- 1.22e-04 1.19e-04	Paternal
NCU21	F	PFIC/BRIC	*ABCB11*	c.386G>A p.C129Y Heterozygous	Likely pathogenic	- Tolerated possibly damaging	- - -	ND
			ABCB11	c.3839T>A p.I1280N Heterozygous	Likely pathogenic	- Deleterious probably damaging	- - -	ND
NCU22	M	PFIC/BRIC	*ABCB11*	c.3121T>C p.Y1041H Heterozygous	Likely pathogenic	- Deleterious probably damaging	- - -	Maternal
			ABCB11	c.3904G>T p.E1302Ter Heterozygous	Pathogenic	DM NA NA	- - -	Paternal
NCU23	M	Unknown with complications	*JAG1*	c.1262G>A p.C421Y Heterozygous	Likely pathogenic	- Deleterious probably damaging	- - -	Maternal
NCU24	F	Unknown with complications	*ABCB11*	c.1709C>T p.A570V Heterozygous	Likely pathogenic	- Deleterious probably damaging	- - -	Paternal
			ABCB11	c.3802C>T p.R1268W Heterozygous	Likely pathogenic	- Deleterious probably damaging	- - 8.92e-06	Maternal
NCU25‡	F	Unknown without complications	*ABCC2*	c.298C>T p.R100Ter Heterozygous	Pathogenic	DM NA NA	- 1.16e-04 2.66e-05	Paternal
			ABCC2	c.2439+2T>C Splice site disruption Heterozygous	Pathogenic	DM NA NA	2.72e-03 1.16e-04 8.89e-06	Maternal
NCU26‡	M	Unknown without complications	*ABCC2*	c.298C>T p.R100Ter Heterozygous	Pathogenic	DM NA NA	- 1.16e-04 2.66e-05	Paternal
			ABCC2	c.2439+2T>C Splice-site disruption Heterozygous	Pathogenic	DM NA NA	2.72e-03 1.16e-04 8.89e-06	Maternal

(continued)

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

Table III. Continued

Subjects	Sex	Clinical diagnosis	Affected gene	Nucleotide change Predicted amino acid change Zygosity	Classification*	HGMD SIFT PolyPhen	MAF HGVB ExAC (ESA) ExAC (Non-ESA)	Parental origin
NCU27	F	Unknown without complications	*ABCC2*	c.1967+2T>C Splice-site disruption Heterozygous	Pathogenic	DM NA NA	1.37e-03 - -	ND
			ABCC2	c.2302C>T p.R768W Heterozygous	Pathogenic	DM Deleterious probably damaging	2.52e-03 3.47e-04 5.33e-05	ND
NCU28	F	Unknown without complications	*SLC25A13*	c.15G>A Splice-site disruption Heterozygous	Pathogenic	DM NA NA	- - -	Maternal
			SLC25A13	c.1177+1G>A Splice-site disruption Heterozygous	Pathogenic	DM NA NA	3.18e-03 1.04e-03 -	Paternal

DM, disease-causing mutation; *ExAC (ESA)*, Exome Aggregation Consortium (East Asian); *ExAC (Non-ESA)*, Exome Aggregation Consortium (Non-East Asian); *F*, female; *HGMD*, Human Gene Mutation Database; *M*, male; *NA*, not applicable; *ND*, not determined; *PolyPhen*, Polymorphism Phenotyping; *SIFT*, Sorting Intolerant From Tolerant; -, no registration data.
Accession number: *JAG1*, NM_000214.2; *NOTCH2*, NM_024408.3; *ABCC2*, NM_000392.3; *SLC25A13*, NM_014251.2; *ATP8B1*, NM_005603.4; *ABCB11*, NM_003742.2.
*According to the American College of Medical Genetics and Genomics interpretation guidelines.[19]
†The mutation in NCU-13 was predicted as a "Broken WT Donor Site" by Human Splicing Finder.
‡Sibling.

FLA 5.4.0 DTD ■ YMPD8082_proof ■ 22 January 2016 ■ 8:53 pm ■ ce JM

nature COMMUNICATIONS

ARTICLE

Received 22 Nov 2014 | Accepted 7 Jul 2015 | Published 21 Aug 2015

DOI: 10.1038/ncomms9018 OPEN

Rare variant discovery by deep whole-genome sequencing of 1,070 Japanese individuals

Masao Nagasaki[1,2,3,*], Jun Yasuda[1,2,*], Fumiki Katsuoka[1,2,*], Naoki Nariai[1,†], Kaname Kojima[1,2], Yosuke Kawai[1,2], Yumi Yamaguchi-Kabata[1,2], Junji Yokozawa[1,2], Inaho Danjoh[1,2], Sakae Saito[1,2], Yukuto Sato[1,2], Takahiro Mimori[1], Kaoru Tsuda[1], Rumiko Saito[1], Xiaoqing Pan[1,†], Satoshi Nishikawa[1], Shin Ito[1], Yoko Kuroki[1,†], Osamu Tanabe[1,2], Nobuo Fuse[1,2], Shinichi Kuriyama[1,2,4], Hideyasu Kiyomoto[1,2], Atsushi Hozawa[1,2], Naoko Minegishi[1,2], James Douglas Engel[5], Kengo Kinoshita[1,3,6], Shigeo Kure[1,2], Nobuo Yaegashi[1,2], ToMMo Japanese Reference Panel Project[#] & Masayuki Yamamoto[1,2]

The Tohoku Medical Megabank Organization reports the whole-genome sequences of 1,070 healthy Japanese individuals and construction of a Japanese population reference panel (1KJPN). Here we identify through this high-coverage sequencing (32.4 × on average), 21.2 million, including 12 million novel, single-nucleotide variants (SNVs) at an estimated false discovery rate of <1.0%. This detailed analysis detected signatures for purifying selection on regulatory elements as well as coding regions. We also catalogue structural variants, including 3.4 million insertions and deletions, and 25,923 genic copy-number variants. The 1KJPN was effective for imputing genotypes of the Japanese population genome wide. These data demonstrate the value of high-coverage sequencing for constructing population-specific variant panels, which covers 99.0% SNVs of minor allele frequency ≥0.1%, and its value for identifying causal rare variants of complex human disease phenotypes in genetic association studies.

[1] Tohoku Medical Megabank Organization, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai 980-8573, Japan. [2] Graduate School of Medicine, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. [3] Graduate School of Information Sciences, Tohoku University, 6-3-09, Aramaki Aza-Aoba, Aoba-ku, Sendai 980-8579, Japan. [4] International Research Institute of Disaster Science, Tohoku University, 468-1, Aramaki Aza-Aoba, Aoba-ku, Sendai 980-0845, Japan. [5] Department of Cell and Developmental Biology, University of Michigan Medical School, 109 Zina Pitcher Place, Ann Arbor, Michigan 48109-2200, USA. [6] Institute of Development, Aging and Cancer, Tohoku University, 4-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. * These authors contributed equally to this work. † Present addresses: Institute for Genomic Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, USA (N.N. & Y.T.); National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan (X.P.); National Center for Child Health and Development, National Medical Center for Children and Mothers Research Institute, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan (Y.K.). # A full list of consortium members appears at the end of the paper. Correspondence and requests for materials should be addressed to M.N. (email: nagasaki@megabank.tohoku.ac.jp) or to M.Y. (email: masiyamamoto@med.tohoku.ac.jp).