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Original Article

Neonatal Dubin-Johnson Syndrome and its Differentiation from Biliary Atresia

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Abstract

Background and Aims: The aim was to determine if liver biochemistry indices can be used as biomarkers to help differentiate patients with neonatal Dubin–Johnson syndrome (nDJS) from those with biliary atresia (BA). ***Methods:*** Patients with genetically-confirmed nDJS or cholangiographically confirmed BA were retrospectively enrolled and randomly assigned to discovery or verification cohorts. Their liver chemistries, measured during the neonatal period, were compared. Predictive values were calculated by receiver operating characteristic curve analysis. ***Results:*** A cohort of 53 nDJS patients was recruited, of whom 13 presented with acholic stools, and 14 underwent diagnostic cholangiography or needle liver biopsy to differentiate from BA. Thirty-five patients in the cohort, with complete biochemical information measured during the neonatal period, were compared with 133 infants with cholangiographically confirmed BA. Total and direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bile acids, alkaline phosphatase, and gamma-glutamyl transferase were significantly lower in nDJS than in BA. In the discovery cohort, the areas under the curve for ALT and AST were 0.908 and 0.943, respectively. In the validation cohort, 13/15 patients in the nDJS group were classified as nDJS, and 10/53 in the BA control group were positive ($p<0.00001$) with an ALT biomarker cutoff value of 75 IU/L. Thirteen of 15 patients were classified as nDJS and none were classified positive in the BA group (13/15 vs. 0/53, $p<0.00001$) with an AST cutoff of 87 IU/L. ***Conclusions:*** Having assembled and investigated the largest cohort of nDJS patients reported to date, we found that nDJS patients could be distinguished from BA patients using the serum AST level as a biomarker. The finding may be clinically useful to spare cholestatic nDJS patients unnecessary invasive procedures.

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Introduction

Hyperbilirubinemia II, or Dubin–Johnson syndrome (DJS; OMIM 237500), was first reported by Dubin and Johnson in 1954.[1] It is an autosomal recessive disorder caused by variations in the ATP-binding cassette subfamily C member 2 (*ABCC2*) gene that result in a decrease in the production or loss of function of multidrug resistance-associated protein 2 (MRP2).[2,3] The disorder presents in adolescence and is characterized by a low-grade elevation of conjugated bilirubin in the blood, and histologically with the accumulation of dark, coarsely granular, melanin-like pigment in centrilobular hepatocytes, with no other signs of hepatic injury.[4,5] It has been reported that DJS can present with severe cholestasis and hepatomegaly in neonates (nDJS).[6–8] However, with only a limited number of reported cases, the clinical symptoms, pathologic signs, and genetic features of nDJS remain poorly described.

Biliary atresia (BA) is the leading cause of pediatric liver transplantation worldwide. It presents as an obliterative cholangiopathy with neonatal jaundice and pale stools.[9] Prompt diagnosis and the Kasai procedure improve the odds of native liver survival. However, differentiation from other causes of neonatal cholestasis is difficult because of the low specificity of imaging studies, including ultrasonography, hepatobiliary iminodiacetic acid scans, and magnetic resonance cholangiopancreatography, among others. Quite often, liver biopsy or surgical cholangiography is needed to confirm the diagnosis.[9]

As nDJS patients usually do not require specific treatment, but BA needs early surgical intervention,[10] early differentiation is critical. A previous study in Japan by Togawa *et al*.,[11] reported that eight of ten nDJS patients underwent liver biopsy during neonatal cholestasis because they were

Keywords: Jaundice; ABCC2; Cholestasis; MRP2; Acholic stools.
Abbreviations: AUC, area under the curve; ABCC2, ATP-binding cassette subfamily C member 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BA, biliary atresia; DB, direct bilirubin; DJS, Dubin–Johnson syndrome; nDJS, neonatal Dubin-Johnson syndrome; GGT, gamma-glutamyl transferase; INR, international normalized ratio; MRP2, multidrug resistance-associated protein 2; ROC, receiver operating characteristic; TB, total bilirubin; TBA, total bile acids.
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suspected of having BA. The primary aim of this study is to better define the clinical and pathologic features of nDJS and to determine whether convenient biomarkers can be identified for helping to distinguish nDJS from BA.

Methods

Subjects

This was a single-center retrospective study. The subjects were Chinese children who were admitted to the Children's Hospital of Fudan University, between 2011 and 2021 for the study of neonatal cholestasis with consent under a protocol (ethical approvals 2015-178 and 2020-402) in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. nDJS was defined as onset of cholestasis before 3 months of age, with confirmed homozygous or compound-heterozygous variants of uncertain significance, or with pathogenic/likely pathogenic *ABCC2* variants, according to American College of Medical Genetics /Association for Molecular Pathology guidelines,[12] and after exclusion of other causes such as infection, hemolytic jaundice, endocrine disease, bile acid synthesis disorders, and other metabolic conditions. Of 53 genetically-confirmed nDJS patients (Table 1), the 35 patients with complete liver biochemistry data obtained during the neonatal period were selected as the test subjects. A group of 133 age- and sex-matched cholangiography-confirmed BA patients, with available pre-operation liver biochemistry indices, were used as the control group. Their liver chemistry indices were compared and analyzed by receiver operating characteristic (ROC) curves. A total of 80 patients with BA and 20 with nDJS were randomized to the discovery cohort; in addition, 53 patients with BA and 15 with nDJS were included in the validation cohort.

The screening criteria for BA included: (1) Onset of clinical manifestations of BA, such as prolonged jaundice with high GGT levels and acholic stools during the neonatal period; (2) A cholangiogram showing that the bile ducts were not patent during the cholangiography; (3) Biopsy specimens evaluated by two pathologists to ascertain the diagnosis of BA; (4) Exclusion of other causes of neonatal cholestasis through appropriate investigations, such as Alagille syndrome, citrin deficiency, progressive familial intrahepatic cholestasis type 3 and other metabolic conditions.

Ninety-four age-matched patients with neonatal intrahepatic cholestasis were chosen as cholestasis controls (Supplementary Table 1). They included four patients with bile acid synthesis defects, three with citrin deficiency, one with progressive familial intrahepatic cholestasis type 2, 21 with Alagille syndrome, and 65 with an unknown etiology, i.e. testing for neonatal hemochromatosis, viral hepatitis, and others. failed to identify an etiology.

Pathogenicity of newly discovered missense ABCC2 variants

The pathogenicity of newly discovered missense variants was predicted by *in silico* tools including the MutationTaster (http://www.mutationtaster.org/), Polymorphism Phenotyping v2 (Polyphen-2, http://genetics.bwh.harvard.edu/pph2/index.shtml), Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org), Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org/index.php), Mendelian Clinically Applicable Pathogenicity (http://bejerano.stanford.edu/MCAP/), and Functional Analysis Through Hidden Markov Models (http://fathmm.biocompute.org.uk/). The pathogenicity of noncanonical splicing variants and non-frameshift insertion/deletion variants was predicted by MutationTaster. Default settings were used for all *in silico* tools.

Liver histopathology

Liver biopsy specimens were obtained from one patient with neonatal Dubin–Johnson syndrome during evaluation of the cause of cholestasis and from seven during laparoscopic cholangiography because BA was suspected. The specimens were processed routinely, and 4 μm sections were cut from formalin-fixed, paraffin-embedded blocks and stained with hematoxylin and eosin for routine histologic evaluation of collagen, bile, and melanin-like pigment deposits in hepatocytes.

Statistical analysis

Statistical analysis was performed with SPSS 19.0 (IBM Corp., Armonk, NY, USA) to determine differences between the nDJS patients and control groups. Kolmogorov-Smirnov tests were performed to check if the data were normally distributed. Student's *t*-test was performed when the data had a normal distribution. A nonparametric test, the Mann-Whitney U test, was performed when the data did not have a normal distribution. Data were reported as medians and interquartile range, or means ± SD. ROC curve analysis was used to calculate the areas under the curve (AUC) with 95% confidence intervals (CIs).

Results

Clinical features of nDJS patients

During the study period, about 7,000 patients were referred to us for investigation of the causes of neonatal cholestasis. Of those 53 (31 men and 22 women) were diagnosed with nDJS (Table 1). Cholestasis subsided or improved during follow-up in all nDJS patients, and none failed to thrive. Of 28 patients with a recorded date of onset of jaundice during the neonatal period, 25 visited a pediatric gastroenterologist because of jaundice shortly after birth (0–10 days after birth, with an average of 3.6±1.9 days. Another three patients presented with jaundice at the first, second, and third month after birth. Peak jaundice was detected at 2–79 (27.9±19.9) days after birth, during the first 3 months. Peak total bilirubin (TB) ranged from 3.5–29.3mg/dL (13.9±6.3mg/dL) and peak direct bilirubin (DB) ranged from 2.3–22.2mg/dL (6.2±4.5mg/dL). Twenty-two patients were followed-up at our center. Jaundice disappeared in seven patients (P7, P13, P20, P21, P22, P32, P41), declined to subclinical levels (TB levels between 1–2mg/dL) in five (P8, P9, P15, P24, P43), and persisted in six (2–4mg/dL; P5, P12, P16, P27, P29, P42) at the last follow-up. Jaundice in patients P3, P17, and P31 disappeared at follow-up but relapsed as subclinical jaundice of unknown cause. P37 experienced a recurrence of subclinical jaundice at 3.2 years of age after a fever lasting for 2 days.

The liver and spleen were palpable 2–3 cm below the costal margin or xiphoid in some cases, but were not palpable in others. Fourteen patients had cholangiography or liver biopsy or both, to verify the presence of BA. Thirteen of 30 patients with descriptions of stool color were reported to have acholic stools. All infants diagnosed with DJS were full-term except for patients P15, P28, and P30 (Table 1). Three (P28, P30, P42) presented with transient hypoglycemia, and three (P6, P34, P40) presented with transient prolonged

Table 1. Clinical and genetic characteristics of the study subjects

P	S	ABCC2 variation	Acholic stools	Jaundice	Liver below the rib	Liver below the xiphoid	Spleen below the rib	Cholangiography	Liver biopsy	Additional observations
1	M	c.1627C>T (p.Gln543Ter); c.3825C>G (p.Tyr1275Ter)	–	+	–	–	–			
2	M	c.3825C>G (p.Tyr1275Ter); c.2755T>A (p.Ser919Thr), c.4239_4240dupTC (p.His1414LeufsTer18)		+						
3	M	c.2303G>A (p.Arg768Gln); c.3476T>G (p.Ile1159Ser)		+						jaundice free after infant, recurrent jaundice at 8.5 years
4	F	c.3825C>G (p.Tyr1275Ter); c.1399G>A (p.Val461Ile)		+	–	–	–			
5	M	c.298C>T (p.Arg100Ter); c.3928C>T (p.Arg1310Ter)		+	–	–	–			persistent jaundice at 10 years
6	M	c.3825C>G (p.Tyr1275Ter); c.1322_1325dupTGTG (p.Trp442CysfsTer37)		+	–	–	–			transient prolonged prothrombin time (international normalized ratio [INR])
7#	M	c.1177C>T (p.Arg393Trp); c.1177C>T (p.Arg393Trp)		+	2 cm	1 cm	–		+	jaundice free at 7.5 months
8#	F	c.1177C>T (p.Arg393Trp); c.4024T>C (p.Ser1342Pro)	–	+	–	–	–			subclinical jaundice at 4.3 months
9#	F	c.1177C>T (p.Arg393Trp); c.4024T>C (p.Ser1342Pro)	+	+	2 cm	–	–	+		persistent jaundice at 6.2 months, subclinical jaundice at 7.3 months and 9.4 months
10	F	c.1815+3_1815+6dup; c.1813delinsCAGGT (p.Ala606ValfsTer17)		+	–	–	–			
11	F	c.1281T>G (p.Asp427Glu); c.4025C>A (p.Ser1342Tyr)		+	–	–	–			
12#	M	c.1177C>T (p.Arg393Trp); c.2980delA (p.Ile994LeufsTer29)		+						persistent jaundice at 8.9 months
13	M	c.3825C>G (p.Tyr1275Ter); c.1177C>T (p.Arg393Trp)		+				+		mother had itching during pregnancy, jaundice free at 11.0 months
14#	M	c.632+2_632+5delTAGG; c.4239_4240dupTC (p.His1414LeufsTer18)		+						
15#	M	c.2302C>T (p.Arg768Trp); c.4024T>C (p.Ser1342Pro)	+	+	4 cm	2 cm	–	+		premature delivery, subclinical jaundice at 7.4 months
16#	M	c.3825C>G (p.Tyr1275Ter); c.4146+1G>T	–	+	2 cm	2 cm	–			persistent jaundice at 2.2 years
17#	F	c.1177C>T (p.Arg393Trp); c.4145A>G (p.Gln1382Arg)		+						jaundice free at 8.0 months, subclinical jaundice at 1.0 year
18#	F	c.2821delG (p.Glu941LysfsTer5); c.4313+1G>T	–	+	3 cm	–	–			low muscle tension

(continued)

Table 1. *(continued)*

P	S	ABCC2 variation	Acholic stools	Jaundice	Liver below the rib	Liver below the xiphoid	Spleen below the rib	Cholangiography	Liver biopsy	Additional observations
19	F	c.1018C>A (p.Pro340Thr); c.1968-1G>A		+	2.5 cm	2.5 cm	–			
20#	M	c.1177C>T (p.Arg393Trp); c.2063T>C (p.Met688Thr)	+	+	2.3 cm	–	2.3 cm	+	+	jaundice free at 5 months
21#	M	c.4250delC (p.Ser1417PhefsTer14); c.4256T>G (p.Val1419Gly)		+						jaundice free at 4.1 months
22	M	c.1177C>T (p.Arg393Trp); c.3660delC (p.Ser1222GlnfsTer3)		+	1 cm	–	–			jaundice free at 4.7 months
23#	F	c.4384G>A (p.Glu1462Lys); c.4384G>A (p.Glu1462Lys)	+	+						
24#	M	c.2755T>A (p.Ser919Thr); c.2883+1G>A, c.4239_4240dupTC (p.His1414LeufsTer18)	–	+						subclinical jaundice at 3.3 years
25#	F	c.1177C>T (p.Arg393Trp); c.1177C>T (p.Arg393Trp)	+	+	3 cm;	2.5 cm	–	+	+	
26#	F	c.3259-1G>A; c.3191T>A (p.Ile1064Asn)		+						
27#	M	c.3825C>G (p.Tyr1275Ter); c.1177C>T (p.Arg393Trp)		+	2 cm	3 cm	–	+	+	persistent jaundice at 4.9 months
28#	M	c.338T>C (p.Leu113Pro); c.338T>C (p.Leu113Pro)	+	+	1 cm	–	–	+	+	premature, hypoglycemia, convulsion, atrial septal defect, retarded brain development
29	M	c.2443C>T (p.Arg815Ter); c.2078G>A (p.Gly693Glu)		+	–	–	–			persistent jaundice at 7.0 months
30#	M	c.1177C>T (p.Arg393Trp); c.1177C>T (p.Arg393Trp)	–	+						premature delivery; hypoglycemia; atrial septal defect
31#	M	c.2302C>T (p.Arg768Trp); c.3988-3C>G, c.1457C>T (p.Thr686Ile)		+	–	–	–			jaundice free at 9.6 months, subclinical jaundice at 2.5y
32#	M	c.2755T>A (p.Ser919Thr); c.4239_4240dupTC (p.His1414LeufsTer18)	+	+	2.5 cm	1.5 cm	–			jaundice free at 1.4 years
33#	F	c.3825C>G (p.Tyr1275Ter); c.2201T>C (p.Leu734Pro)	+	+						
34#	M	c.2366C>T (p.Ser789Phe); c.2362_2363delCT (Leu788ValfsTer13)	–	+						transient prolonged prothrombin time (INR)
35	M	c.4239_4240dupTC (p.His1414LeufsTer18), c.1462C>T (p.Gln488Ter), c.2755T>A (p.Ser919Thr)	+	+	4 cm	3.5 cm	–			
36	F	c.4384delG (p.Glu1462ArgfsTer8); c.298C>T (p.Arg100Ter)		+	–	–	–	+		N/A

(continued)

Table 1. *(continued)*

P	S	ABCC2 variation	Acholic stools	Jaundice	Liver below the rib	Liver below the xiphoid	Spleen below the rib	Cholangiography	Liver biopsy	Additional observations
37	M	c.2026G>C (p.Gly676Arg); c.1939G>T (p.Glu647Ter)	+	+	–	–	–		+	jaundice free after infancy, recurrent subclinical jaundice at 3.2 years after fever
38#	F	c.3825C>G (p.Tyr1275Ter), c.2755T>A (p.Ser919Thr), c.4239_4240dupTC (p.His1414LeufsTer18)		+	–	–	–			
39	F	c.3928C>T (p.Arg1310Ter); c.1882C>T (p.Arg628Ter)	–	+	4 cm	2.5 cm	–			
40#	F	c.1177C>T (p.Arg393Trp); c.1177C>T (p.Arg393Trp)	+	+	2.5 cm	3.5 cm	2 cm	+		transient prolonged prothrombin time (INR)
41#	F	c.338T>C (p.Leu113Pro); c.2855_2858delTTAA (p.Ile952ArgfsTer5)		+	3 cm	–	–			jaundice free at 5.1 months
42#	F	c.4239_4240dupTC (p.His1414LeufsTer18); c.2755T>A (p.Ser919Thr), c.4343G>A (p.Gly1448Asp)	–	+	1.5 cm	3	–			hypoglycemia, persistent jaundice at 7.5 months
43#	F	c.1177C>T (p.Arg393Trp); c.2980delA (p.Ile994LeufsTer29)		+	–	–	–	+	+	subclinical jaundice at 3.9 months
44#	F	c.3825C>G (p.Tyr1275Ter); c.2077G>A (p.Gly693Arg)	–	+	2 cm	–	–			
45#	F	c.3928C>T (p.Arg1310Ter); c.116delA (p.Tyr39SerfsTer40)	–	+	2 cm	2 cm	–			
46#	M	c.1939G>T (p.Glu647Ter); c.4384delG (p.Glu1462ArgfsTer8)	–	+	3 cm	–	–			
47#	F	c.3196C>T (p.Arg1066Ter); c.2439+5G>A	–	+	2 cm	–	–			
48#	M	c.3825C>G (p.Tyr1275Ter); c.3825C>G (p.Tyr1275Ter)	–	+	–	–	–			
49	M	c.4465_4473delinsGGCCCACAG (p.Ile1489_ Ile1491delinsGlyProGln); c.2302C>T (p.Arg768Trp)	–	+	2.5cm	2.5 cm	–			
50#	M	c.389G>A (p.Trp130Ter); c.3196C>T (p.Arg1066Ter)	+	+	2.5 cm	2 cm	–			
51	M	c.1939G>T (p.Glu647Ter); c.3928C>T (p.Arg1310Ter)	–	+	–	–	–			
52#	M	c.2830G>T (p.Glu944Ter); c.4465_4473delinsGGCCCACAG (p.Ile1489_Ile1491delinsGlyProGln)	–	+	2 cm	–	2 cm			
53#	M	c.1078G>T (p.Gly360Ter), c.4239_4240dupTC (p.His1414LeufsTer18), c.2755T>A (p.Ser919Thr)	+	+	3 cm	4 cm	–	+	+	

P, patient; S, sex; M, male; F, female; –, negative; +, positive. #Patients with liver function data during neonatal period (see Table 3, and Figs. 1 and 3).

prothrombin time responsive to vitamin K1 injection. One patient (P18) had low muscle tension. The mother of P13 reported itching during pregnancy, and P28 had retarded brain development. The characteristics of BA patients and cholestasis controls are shown in Supplementary Material 1.

ABCC2 variants

Fifty-five *ABCC2* variants were identified in 53 nDJS patients (Table 1). c.1177C>T (p.Arg393Trp), c.3825C>G (p.Tyr1275Ter), c.2755T>A (p.Ser919Thr), c.4239_4240dupTC (p.His1414LeufsTer18), c.3928C>T (p.Arg1310Ter) occurred in seventeen, twelve, seven, seven, and four alleles, respectively; c.1939G>T (p.Glu647Ter), c.2302C>T (p.Arg768Trp), c.338T>C (p.Leu113Pro), c.4024T>C (p.Ser1342Pro) were each found in three alleles, while c.2980delA (p.Ile994LeufsTer29), c.298C>T (p.Arg100Ter), c.3196C>T (p.Arg1066Ter), c.4384delG (p.Glu1462ArgfsTer8), c.4384G>A (p.Glu1462Lys), c.4465_4473delinsGGCCCACAG (p.Ile1489_Ile1491delinsGlyProGln) occurred two times each.

Identification of novel ABCC2 variants and in silico assessment

Twenty-two of the variations we observed had been previously reported,[10,11,13–16] and thirty-three were novel (Table 2). Of the novel variants, six were canonical splicing variants, two were noncanonical splicing variants, five were nonsense variants, thirteen were missense variants, and seven were frameshift variants. No significant correlations were observed between the type of variant and the presence of acholic stools (Supplementary Material 2, Supplementary Table 2).

Biochemical features

Total protein and albumin values were within the normal range in DJS patients (data not shown). We evaluated serum TB, DB, total bile acids (TBAs), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) in 35 patients during neonatal cholestasis between 16 and 93 days of age. In those nDJS patients the median and interquartile range was determined for TB [7.2 (5.3, 9.1) mg/dL], DB [4.5 (3.3, 5.8) mg/dL], ALT [29.7 (16.5, 45) IU/L], AST [33.5 (23.1, 68.8) IU/L], ALP [386 (326, 524) IU/L], GGT [149 (84.5, 216.5) IU/L], and TBA [72.2 (30.6, 109.1) μmol/L]. TB, DB, ALT, AST, GGT, ALP, and TBA levels were all significantly lower in infants with nDJS than in infants with BA. DB, ALT, AST, and ALP levels were also significantly lower in nDJS patients than in cholestasis controls (Fig. 1). No significant correlations were observed between the type of variant and the levels of TB, DB, ALT, and AST (Supplementary Material 2, Supplementary Fig. 1). In addition, TB, DB, ALT, AST, TBA, ALP, and GGT levels had no significant linear correlation with days after birth in any of the patient groups. (Supplementary Fig. 2).

Pathological findings

Liver biopsy specimens were obtained from eight patients at 1–3 months of age, six of whom had undergone cholangiography and one with a needle liver biopsy to verify the presence of BA. Only one patient was reported to have a dark red liver during the procedure, at 40 days of age. Histopathologically, the presence of fibrosis, cirrhosis, or melanin-like pigment deposits in hepatocytes was not observed, except fibrosis in P20. However, giant-cell transformation of hepatocytes/hepatocyte ballooning (6/8), steatosis (4/8), and slight intracanalicular and intracytoplasmic cholestasis, i.e. bile plugs (7/8) were observed. The results of five patients with liver biopsies at our center are shown in Figure 2.

ROC curve analysis

Performance of discriminatory features of biomarkers for nDJS diagnosis: 35 nDJS patients and 133 age-matched subjects with BA were enrolled in the study (Supplementary Fig. 3). To investigate the predictive values of discriminatory features (TB, DB, TBA, ALT, AST, GGT, and ALP levels) for nDJS, we calculated the AUC of each feature in the discovery cohort (Fig. 3). The AUC for ALT in nDJS was 0.908 (95% CI: 0.834–0.982; $p<0.0001$) and that of AST was 0.943 (95% CI: 0.861–1.000; $p<0.0001$; Fig. 3E). The sensitivity and specificity for ALT were 81.25% and 90%, respectively, at a cutoff value 75 IU/L. The optimal threshold cutoff for AST in nDJS, was 87 IU/L with a sensitivity of 95% and a specificity of 90%. We tested the discriminating power of ALT and AST using a double-blind strategy in a validation cohort of 68 subjects, 15 with DJS and 53 with BA (Table 3). Thirteen of the 15 nDJS were classified as positive and 10 of the 53 BA patients were classified as positive using ALT as the biomarker. With an AST cutoff value of <87 IU/L, 13 of 15 patients were classified as positive in the nDJS group, and 0 of the 53 BA patients were classified as positive.

Discussion

Accurate diagnosis of DJS in neonates is important to rule out other hepatobiliary disorders such as BA, which is the most common cause of neonatal cholestasis and usually progresses rapidly, with severe liver injury leading to liver transplantation.[5,10] nDJS is a relatively mild, autosomal recessive disorder that does not develop into severe liver injury, but manifests with severe cholestasis and acholic stools during the neonatal period, a presentation that sometimes overlaps with BA. The Kasai hepatic portoenterostomy procedure for re-establishing bile flow is the most effective surgical intervention for survival in BA. The liver is preserved, and the procedure is time-sensitive, with best outcomes if performed before 60 days of age.[17] Thus, patients suspected of BA often undergo invasive liver biopsy/cholangiography in order to obtain a clear diagnosis. However, the similarity in presentation between nDJS and BA, and the importance of a successful differential diagnosis, has not attracted much attention and is not a topic discussed in the guidelines for evaluating cholestatic jaundiced infants by the North American and European Societies for Pediatric Gastroenterology, Hepatology, and Nutrition.[18] The lack of diagnostic differentiation often leads to nDJS patients being sent for liver biopsy to exclude BA, as shown in our study cohort and in a previous Japanese study cohort.[11]

To address that issue, we assembled the largest cohort of nDJS patients reported to date at our center to characterize their clinical symptoms, liver chemistries, and pathologic and genetic features. We investigated whether a convenient, noninvasive, biomarker for the early diagnosis of nDJS, differentiating it from BA, could be found. We report here that the liver biochemistry indices of nDJS were generally lower than those in BA. ROC curve analysis of various enzymes revealed that serum ALT and AST levels were robust differentiators of DJS and BA in neonates. The use of an AST cutoff of <87 IU/L was particularly helpful in distin-

Table 2. Allele frequencies and *in silico* identification of 33 *ABCC2* variants

cDNA change (NM_000392.5)	Protein change (NP_000383.2)	gnomAD	gnomAD_EAS	MuT	SIFT	PPH2	PROVE-AN	M-CAP	FATH-MM	ACMG
c.338T>C	p.Leu113Pro	0.00004950 (0/4/282,822)	0.0007016 (0/14/19,954)	D	D	D	D	D	D	VUS
c.389G>A	p.Trp130Ter	0.000003978 (0/1/251,358)	0(0/0/18,394)	NMD	/	/	/	/	/	P
c.632+2_632+5delTAGG	/	–	–	D	/	/	/	/	/	LP
c.1018C>A	p.Pro340Thr	–	–	D	D	D	D	D	D	VUS
c.1078G>T	p.Gly360Ter	–	–	NMD	/	/	/	/	D	P
c.1281T>G	p.Asp427Glu	–	–	D	D	D	D	D	D	VUS
c.1322_1325dupTGTG	p.Trp442CysfsTer37	–	–	NMD	/	/	/	/	/	P
c.1399G>A	p.Val461Ile	0.000003981 (0/1/251,204)	0.00005440 (0/1/18,384)	D	N	N	N	D	D	VUS
c.1462C>T	p.Gln488Ter	–	–	NMD	/	/	/	/	/	P
c.1627C>T	p.Gln543Ter	–	–	NMD	/	/	/	/	/	P
c.1813delinsCAGGT	p.Ala606ValfsTer17	–	–	NMD	/	/	/	/	/	P
c.1815+3_1815+6dup	/	–	–	D	/	/	/	/	/	LP
c.1968-1G>A	/	0.000003979 (0/1/251,348)	0(0/0/18,392)	D	/	/	/	/	/	P
c.2063T>C	p.Met688Thr	–	–	D	D	D	D	D	D	VUS
c.2821delG	p.Glu941LysfsTer5	–	–	NMD	/	/	/	/	/	P
c.2830G>T	p.Glu944Ter	–	–	NMD	/	/	/	/	/	P
c.2883+1G>A	/	–	–	D	/	/	/	/	/	P
c.2980delA	p.Ile994LeufsTer29	–	–	NMD	/	/	/	/	/	P
c.3191T>A	p.Ile1064Asn	–	–	D	N	D	D	D	D	VUS
c.3259-1G>A	/	–	–	D	/	/	/	/	/	P
c.3476T>G	p.Ile1159Ser	–	–	D	D	D	D	D	D	VUS
c.3660delC	p.Ser1222GlnfsTer3	–	–	NMD	/	/	/	/	/	P
c.3988-3C>G	/	–	–	D	/	/	/	/	/	P
c.4024T>C	p.Ser1342Pro	–	–	D	D	D	D	D	D	VUS
c.4025C>A	p.Ser1342Tyr	–	–	D	D	D	D	D	D	VUS
c.4146+1G>T	/	–	–	D	/	/	/	/	/	P
c.4238_4239dupCT	p.His1414LeufsTer18	–	–	NMD	/	/	/	/	/	P
c.4256T>G	p.Val1419Gly	–	–	D	D	D	D	D	D	VUS
c.4313+1G>T	/	–	–	D	/	/	/	/	/	P
c.4343G>A	p.Gly1448Asp	–	–	D	D	D	N	D	D	VUS
c.4384delG	p.Glu1462ArgfsTer8	–	–	NMD	/	/	/	/	/	P
c.4384G>A	p.Glu1462Lys	–	–	D	D	D	D	D	D	VUS
c.4465_4473delins GGCCCACAG	p.Ile1489_ Ile1491delinsGlyProGln	–	–	D	/	/	/	/	/	LP

ACMG: American College of Medical Genetics and Genomics; FATHMM, Functional Analysis Through Hidden Markov Models (http://fathmm.biocompute.org.uk/); –, not reported/not applicable; D, disease-causing; gnomAD and gnomAD_EAS, allele frequencies of corresponding variants in all populations and in East Asian populations in gnomAD (http://gnomad-old.broadinstitute.org/), respectively; LP, likely pathogenic variants; M-CAP, Mendelian Clinically Applicable Pathogenicity (http://bejerano.stanford.edu/MCAP/); MuT: MutationTaster (http://www.mutationtaster.org); N, no effect; na, not available; NMD, nonsense-mediated mRNA decay; P, pathogenic; PPH2: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml); PROVEAN: Protein Variation Effect Analyzer (http://provean.jcvi.org/index.php); SIFT: Sorting Intolerant From Tolerant (http://provean.jcvi.org/index.php); VUS, variants of uncertain significance.

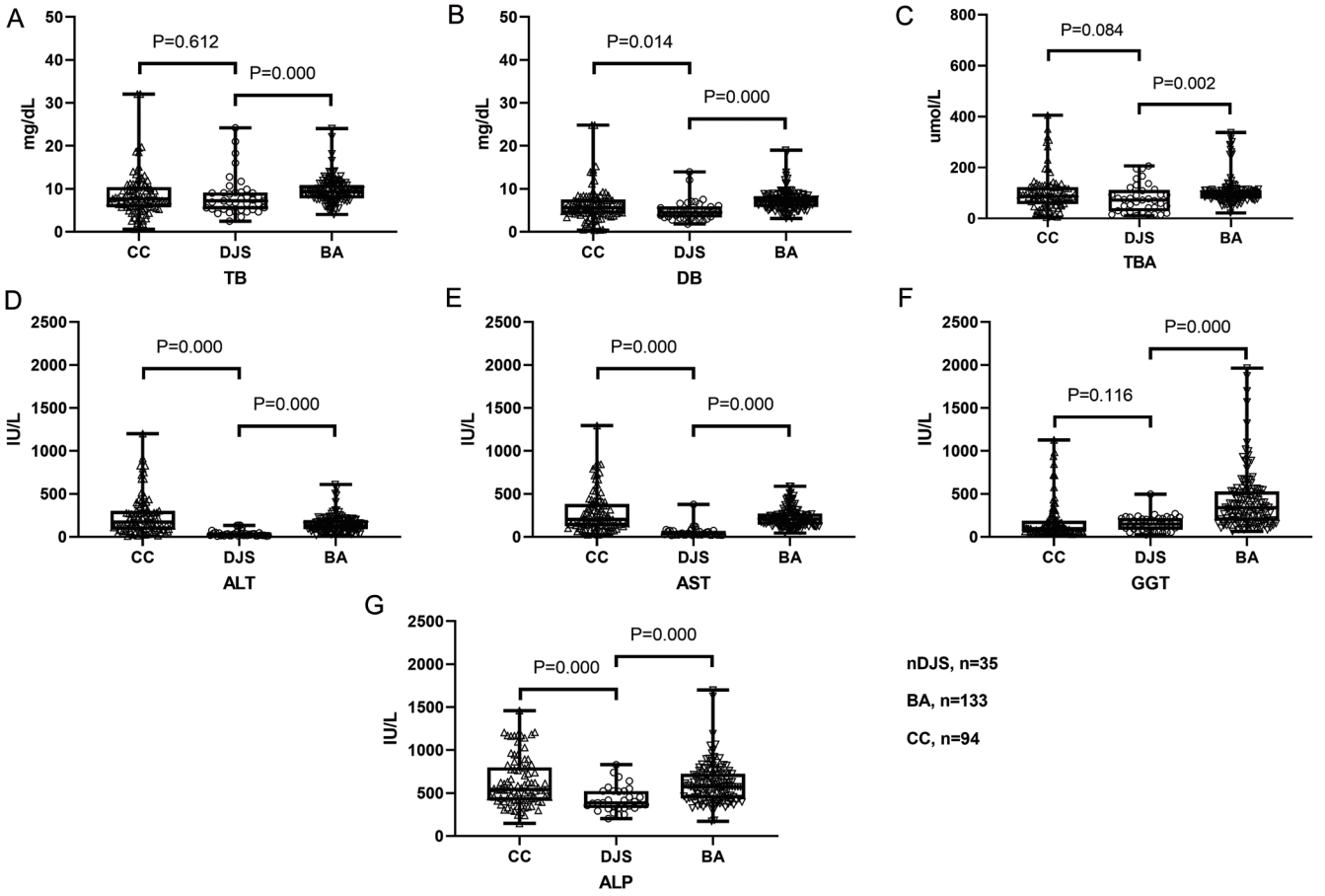

Fig. 1. Biochemical features of neonatal Dubin-Johnson syndrome (nDJS) patients. Total bilirubin (TB), direct bilirubin (DB), total bile acids (TBA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) values in 53 neonatal DSJ patients were compared with 133 biliary atresia (BA) patients and 94 cholestasis controls.

guishing DJS from BA (13/15 nDJS vs. 0/53 BA patients). The finding has potential implications for clinical practice, as liver chemistries are routine and rapid. We believe that such information may be particularly useful in community clinics and nontertiary centers to spare patients invasive procedures such as liver biopsy or cholangiography for diagnosis when nDJS is suspected.[5] This study found that an invasive cholangiography can be avoided for the differentiation of BA in patients with cholestasis, acholic stools and AST levels consistently <80 IU/L. We recognize that elevation of ALT and AST can be affected by a variety of factors, including infection, alcohol, and drugs.[19,20] AST levels might also be affected by an improper blood collection process or hemolysis. Caregivers must pay attention to those factors when using ALT and AST as biomarkers for the diagnosis of nDJS. Only Chinese patients were enrolled in this study. It would be of interest to determine if the observations made in this report are seen in children from other ethnic groups.

It should be noted that increased urinary excretion of coproporphyrin isomer I is a characteristic feature of adult DJS[21] and has been proposed by Junge and Norman[10] as a potential biomarker to differentiate nDJS from BA. They concluded that urinary coproporphyrin analysis is a fast and reliable diagnostic tool for differentiating nDJS from BA in a comparison of four nDJS patients and twenty-six BA patients. We did not include a urinary coproporphyrin isomer in this study since because we did not have access to a commercial test. The same was true for the 10 nDJS patients reported from the study in Japan.[11]

Acholic stools are an important diagnostic feature of BA, but their presence in nDJS patients has also been reported by several centers.[11,15,22] In a previous study in Japan, eight of ten nDJS patients underwent a liver biopsy because of a suspicion of BA.[11] In this study, 13 of the 30 patients, with a described stool color, were reported as having acholic stools, indicating that acholic stool is a relatively common feature of nDJS. Our data suggest that acholic stools are not a reliable differentiator of nDJS and BA.

In adult patients, black livers, or melanin-like pigment. are typical histological characteristics of DJS, but not all nDJS patients present with black livers or melanin-like pigment.[11,14,23] In this study, no melanin-like pigments were observed in any of the eight patients we biopsied, which indicates that a black liver, or accumulation of melanin-like pigment deposits in hepatocytes, is not a reliable characteristic in nDJS patients. That might be because the infants were too young for accumulation of the pigments, as confirmed by a reported case with prolonged cholestasis in early infancy, and an accumulation of specific pigment granules in the liver cells by 6 years of age.[23] The absence of histological accumulation of melanin-like pigment increases the difficulty of diagnosing DJS during the neonatal period.

This study reports 33 new DJS variants among the 55 identified in the patients. That substantially expands the current ABCC2 mutation spectrum. Some alleles [c.1177C>T (p.Arg 393Trp), c.3825C>G (p.Tyr1275Ter), c.4239_4240dupTC (p.

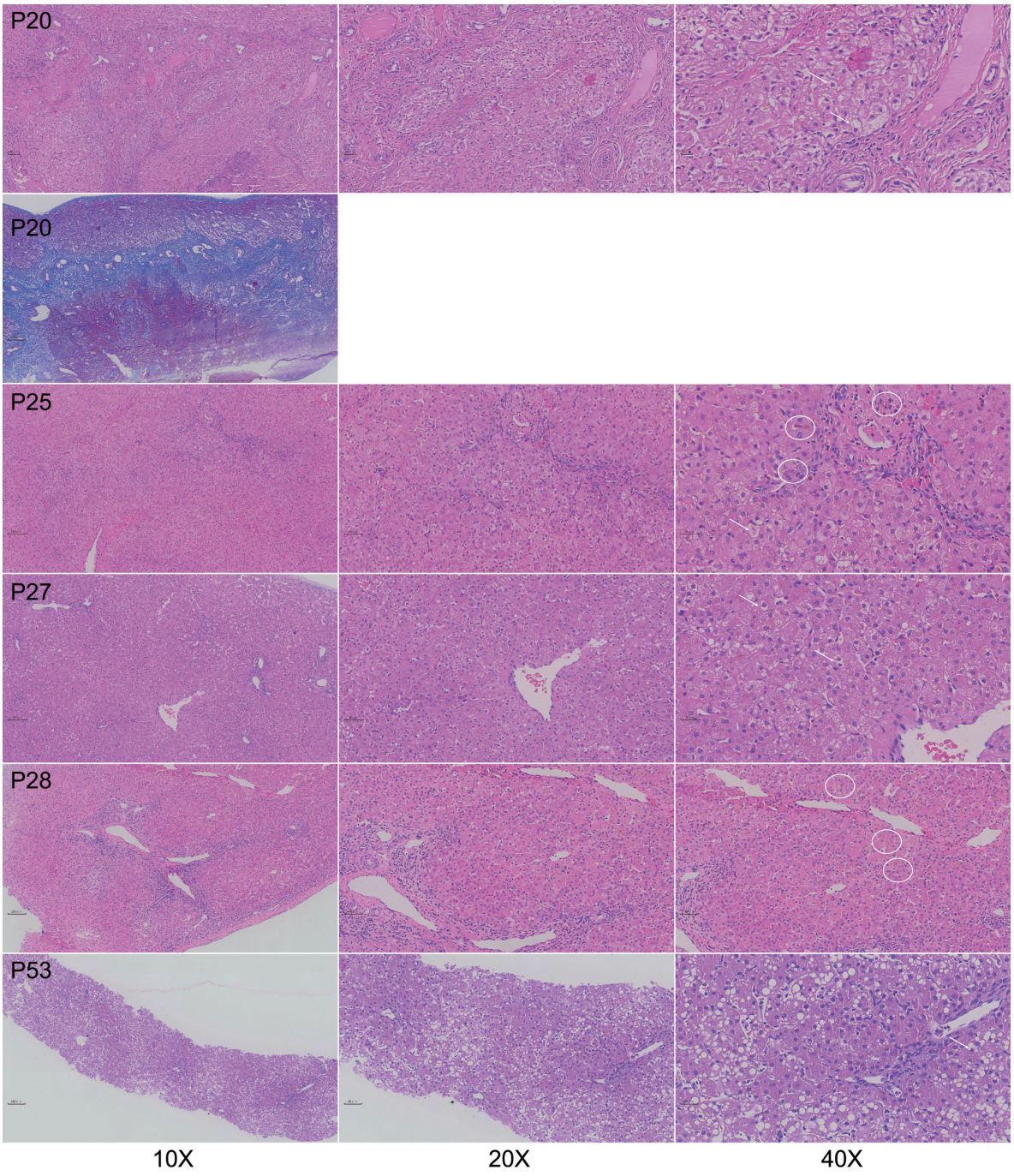

Fig. 2. Histopathologic findings in the livers of five patients. Hematoxylin and eosin staining showed giant-cell transformation of hepatocytes and hepatocyte ballooning, steatosis (arrow) and slight intracanalicular and intracytoplasmic cholestasis with bile plugs, circles). Presence of inflammation was observed in P20, as confirmed by Masson staining (line 2). No inflammation, fibrosis, cirrhosis, or melanin-like pigment deposits in hepatocytes were observed in P25, P27, P28, and P53.

His1414LeufsTer18), c.2755T>A (p.Ser919Thr), c.3928C>T (p.Arg1310Ter), c.298C>T (p.Arg100Ter), c.3196C>T (p.Arg 1066Ter), c.2302C>T (p.Arg768Trp), c.1939G>T (p.Glu647 Ter)] occurred multiple times, suggesting that they are frequent variants in the population. No significant correlations of patient genotype and the presence of acholic stools were found in the study cohort (Supplementary Material 2). It is reasonable to assume that there exists some other factors affecting the disease phenotype. In addition, we observed some novel clinical features in some patients, including three with transient hypoglycemia and three with transient prolonged prothrombin time (INR), which might be worthy of future investigation.

In conclusion, we assembled the largest reported cohort of neonatal Dubin–Johnson syndrome patients that has been evaluated. The most likely negative outcome of their condition is invasive and unnecessary testing to exclude a diagnosis of BA. For that reason, we explored the diagnostic

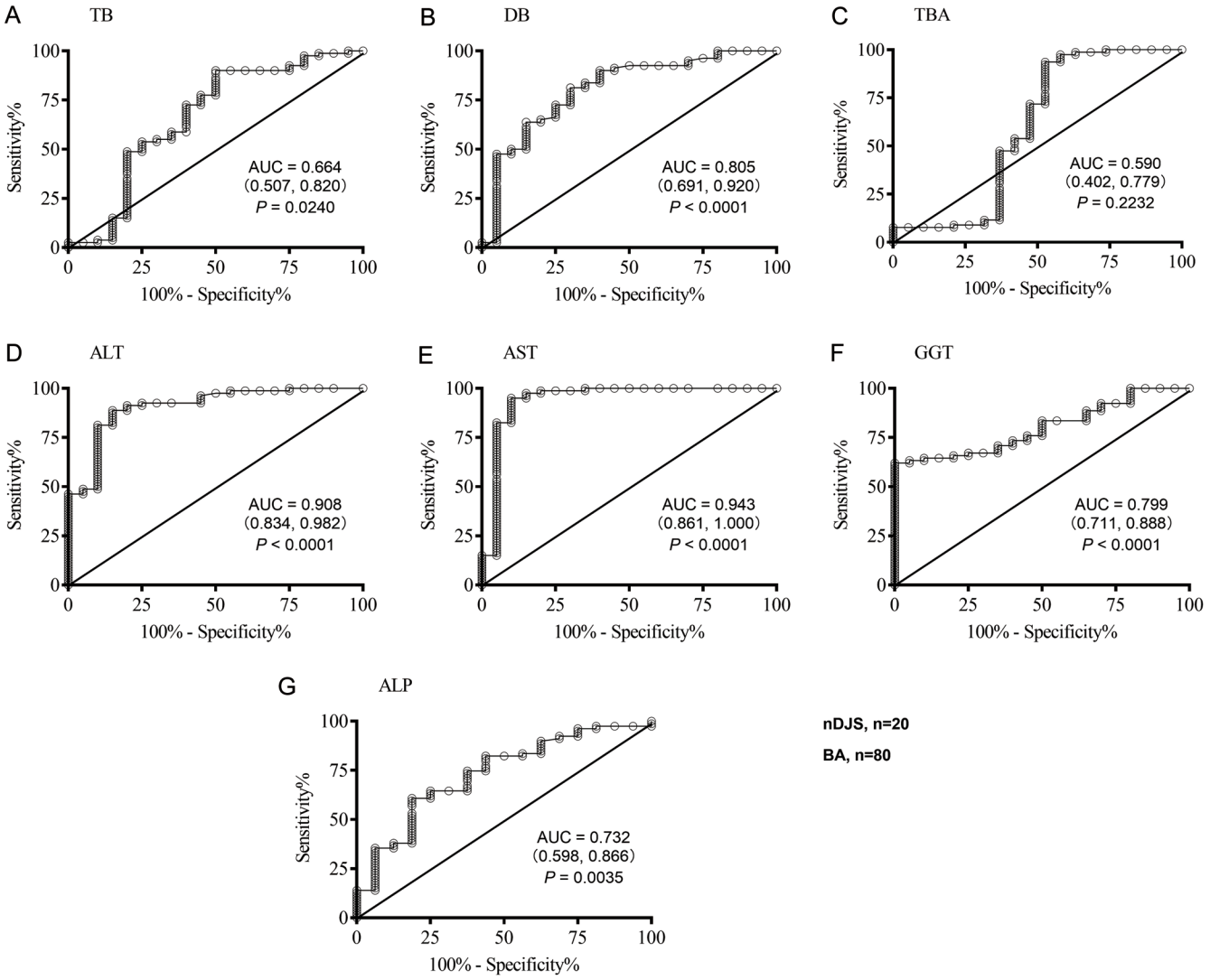

Fig. 3. Receiver operating characteristic curve analysis of discriminatory features in 20 neonatal Dubin-Johnson syndrome (nDJS) patients and 80 biliary atresia (BA) patients in the discovery cohort. The areas under the curve (AUC) for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are >0.9 for differentiating nDJS from BA. The AUCs are shown with 95% CIs.

value of liver biochemistry, which is convenient and routine, and found that serum AST level has the potential to act as a sensitive biomarker for the differentiation of suspected nDJS from a possible misdiagnosis of BA in neonates. A formal diagnosis of nDJS would then follow once confirmatory DNA sequencing at the *ABCC2* locus was carried out.

Table 3. Performance of ALT (cutoff value <75 U/L) and AST (cutoff value <87 IU/L) in patients in the validation cohort with nDJS and BA

	Positive	Negative	Total	
ALT				
nDJS	13	2	15	
BA	10	43	53	$p<0.00001$
AST				
nDJS	13	2	15	
BA	0	53	53	$p<0.00001$

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, biliary atresia; nDJS, neonatal Dubin–Johnson syndrome.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception and design of experiments (JSW, TL), performance of experiments and drafting of the manuscript (TL, JYF, JH, RXW), critical revision of the manuscript (JSW, VL, RXW, JAS), collection of data and samples (TL, JZ, YL), performance of histology analysis (JYF), and data analysis (TL).

Data sharing statement

The detailed data used to support the findings of this study are available from the corresponding author at liuteng@fudan.edu.cn upon request.

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