Variants Associated with Infantile Cholestatic Syndromes Detected in Extrahepatic Biliary Atresia by Whole Exome Studies: A 20-Case Series from Thailand

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Abstract

Biliary atresia (BA) is the most severe form of obstructive cholangiopathy occurring in infants. Definitive diagnosis of BA usually relies on operative findings together with supporting pathological patterns found in the extrahepatic bile duct. In infancy, overlapping clinical patterns of cholestasis can be found in other diseases including biliary hypoplasia and progressive familial intrahepatic cholestasis. In addition, BA has been reported as a phenotype in some rare genetic syndromes. Unlike BA, other cholangiopathic phenotypes have their own established genetic markers. In this study, we used these markers to look for other cholestasis entities in cases diagnosed with BA. DNA from 20 cases of BA, diagnosed by operative findings and histopathology, were subjected to a study of 19 genes associated with infantile cholestasis syndromes, using whole exome sequencing. Variant selection focused on those with allele frequencies in dbSNP150 of less than 0.01. All selected variants were verified by polymerase chain reaction–direct sequencing. Of the 20 cases studied, 13 rare variants were detected in 9 genes: 4 in *JAG1* (Alagille syndrome), 2 in *MYO5B* (progressive familial intrahepatic cholestasis [PFIC] type 6), and one each in *ABCC2* (Dubin–Johnson syndrome), *ABCB11* (PFIC type 2), *UG1A1* (Crigler–Najjar syndrome), *MLL2* (Kabuki syndrome), *RFX6* (Mitchell–Riley syndrome), *ERCC4* (Fanconi anemia), and *KCNH1* (Zimmermann–Laband syndrome). Genetic lesions associated with various cholestatic syndromes detected in cases diagnosed with BA raised the hypothesis that severe inflammatory cholangiopathy in BA may not be a distinct disease entity, but a shared pathology among several infantile cholestatic syndromes.

Keywords
- biliary atresia
- progressive familial intrahepatic cholestasis
- JAG1

Introduction

Biliary atresia (BA) is an inflammatory cholangiopathy of infancy characterized by progressive fibrosclerosis and obliteration of extrahepatic and intrahepatic bile ducts.[1] Clinical manifestations of infants with BA include pale stool, jaundice, and congested liver.[2] If untreated, it ultimately leads to cirrhosis, liver failure, and death within a few years of life. There is no single "gold standard" diagnostic test for BA. Definitive diagnosis is usually made by operative findings of

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collapsed gallbladder, an obliterated extrahepatic biliary tree, and supportive pathological evidence of dense fibrous tissue within the common hepatic duct at the porta hepatis and periportal fibrosis of the liver.[3]

The genetic causes of BA are not clear. As BA is a rare phenotype that potentially leads to early mortality if untreated, the genetic model that best fits this type of congenital anomaly is a polygenic trait with phenotypic modifiers. Previous researches have predominantly focused on discovering a causative gene for the disease. One plausible approach was a genome-wide association study (GWAS), from which single-nucleotide polymorphisms (SNPs) within the intronic regions of *ADD3* were found to have disease association with sporadic BA. Although the association could be validated in various populations including Thais, a fine mapping study did not find significant mutations within the coding elements.[4] One of the limitations of the GWAS approach is that the platform deals with common variants rather than rare mutations.

Associated anomalies of the spleen and the portal vein can be found in BA, which are collectively known as BA splenic malformation syndrome.[5] In addition, there are several genetic syndromes which can have BA as a clinical feature,[6] including Mitchell–Riley syndrome,[7] Fanconi anemia complementation group Q,[8] Zimmermann–Laband syndrome 1,[9] Alagille syndrome (AGS),[10,11] and Kabuki syndrome 1.[12,13] Other bile duct anomalies that share the clinical manifestation of obstructive jaundice with BA are progressive familial intrahepatic cholestasis (PFIC) and biliary hypoplasia (BH). PFIC is a group of rare inherited diseases that cause severe cholestatic liver disease, usually manifest in the first year of life and can progress to lethal liver cirrhosis. The molecular pathogenesis of PFIC is defective bile acid transport caused by mutations involving the transporting mechanisms. To date, there are five types of PFIC recorded in the Online Mendelian Inheritance in Man (OMIM) database.[14–18] Recently, an additional type of PFIC caused by mutations of *MYO5B* was proposed.[19] In East Asians, neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) has a relatively high incidence.[20–22] Apart from these syndromes, there are syndromes that manifest hyperbilirubinemia in infants without structural pathology of the bile ducts, including Crigler–Najjar syndrome,[23] Gilbert syndrome,[24] Dubin–Johnson syndrome,[25] and Rotor syndrome.[26] Although the syndromes described above have distinct clinical characteristics and genetics, they share the same features of cholestasis and many of their associated gene functions as bilirubin transporters. The possibility that BA cases share the same germline mutations as found in these syndromes has not been explored.

In this study, we looked for rare genetic variants in genes previously annotated in various infantile cholestatic syndromes using whole exome sequencing in 20 cases diagnosed with BA. All cases were demonstrated to have an obstructed extrahepatic biliary system and histopathology of the bile duct at the porta hepatis consistent with BA.

Materials and Methods

Patients

DNA samples from liver biopsy tissue of 20 infants with BA who underwent hepatic portoenterostomy (Kasai's operation) at our institute between the years 2003 and 2016 were retrieved from the biological bank under permission from the Research Ethics Committee. All diagnoses of BA were based on operative findings of an obliterated biliary tree at the porta hepatis with or without intraoperative cholangiography. All cases had a clinical history and histopathology compatible with BA. Before the operations, all patients were evaluated by liver function test and liver ultrasonography. A diisopropyl iminodiacetic acid scan was performed when the ultrasonography result was inconclusive.

DNA extraction used a Qiagen DNA Mini kit (Qiagen, Hilden, Germany). Specimens were evaluated for their DNA quality by Nanodrop 2000 spectrophotometry (Thermo Scientific, Delaware, United States) and agarose gel electrophoresis.

Whole Exome Sequencing and In-Silico Analysis

Targeted exonic regions were captured with an Agilent SureSelect XT Human All Exon v5 kit (Agilent Technologies, Santa Clara, California, United States). Paired-end exome sequencing was performed on an Illumina HiSeq-2000 (Illumina, San Diego, California, United States) platform with 100-bp runs at an average mean target depth of 100× coverage.

The raw sequence data in the FASTQ format were mapped to the reference human genome (Homo Sapians GRCh38/hg7) using the Lasergene 15.0 Bioinformatic Suits (DNAstar, Wisconsin, United States). Variant annotation used dbSNP (dbSNP 150). Quality filtering for variant selection used the criteria of SNP percentage at 30% or more of the total read depth and minimum depth at 20×.

Variants Selection and Validation

The genes focused on are shown in ►**Table 1**. The genes included were: Category I: genes from clinical syndromes reported to have BA as a part of their phenotype; Category II: cholangiopathy disorders that can manifest cholestatic jaundice early in life; and Category III; syndromes that manifest with hyperbilirubinemia irrespective of unconjugated or conjugated type. Selection of the Category I genes was made using the OMIM database with the search term "biliary atresia." Because of the considerable incidence of NICCD reported in Asians,[20–22] the condition is included into Category II. Variant annotations used the Seqman Pro program (DNAStar). Selection focused on rare nonsynonymous variants that had minor allele frequencies less than 0.01 in the dbSNP 150 and had variant allele frequency in the ExAC database not greater than 0.01. All selected variants were checked for previous reports suggesting that the alterations were pathogenic, using the Human Gene Mutation Database (University of Cardiff, United States) and NCBI: ClinVar (available from https://www.ncbi.nlm.nih.gov/clinvar/). Variants without prior reported pathological data were predicted for their effects on protein functions by using the Sorting Tolerant From Intolerant (SIFT),[27,28]

Table 1 Genes focused on in this study

Syndrome or disorder	OMIM	Associated gene (chromosome)	Reference
Category I: Syndromes in which BA is reported as one of the phenotypes			
Mitchell–Riley syndrome (MTCHR)	#615710	*RFX6* (6q22.1)	Mitchell et al, 2004
Fanconi anemia, complementation group Q (FANQ)	#612272	*ERCC4* (16p13)	Bogliolo et al, 2013
Zimmermann–Laband syndrome 1 (ZLS1)	#135500	*KCNH1* (1q32)	Balasubramanian and Parker, 2010
Kabuki syndrome 1 (KABUK1)	#147920	*MLL2* (12q13)	McGaughran et al, 2000 and van Haelst et al, 2000
Alagille syndrome 1 (ALGS1)	#188450	*JAG1* (20p12)	Raymond et al, 1989
Alagille syndrome 2 (ALGS2)	#610205	*NOTCH2* (1p12)	McDaniell et al, 2006
Category II: Cholangiopathy disorders that can manifest as cholestatic jaundice in infants			
PFIC type 1 (Byler disease)	#211600	*ATP8B1* (18q21)	Klomp et al, 2004
PFIC type 2	#601841	*ABCB11* (2q31)	Pawlikowska et al, 2010
PFIC type 3	#602347	*ABCB4* (7q21)	de Vree et al, 1998
PFIC type 4	#615878	*TJP2* (9q21)	Sambrotta et al, 2014
PFIC type 5	#617049	*NR1H4* (12q23)	Gomez-Ospina et al, 2016
PFIC type 6	#617049	*MYO5B* (18q21)	Gonzales et al, 2017
Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD)	#605814	*SLC25A13* (7q21)	Ohura et al, 2001 Togawa et al, 2016
Category III: Syndromes with hyperbilirubinemia as their phenotype			
Crigler–Najjar syndrome type II (HBLRCN2)	#606785	*UGT1A1* (2q37)	Moghrabi et al, 1993
Crigler–Najjar syndrome type I (HBLRCN1)	#218800	*UGT1A1* (2q37)	Moghrabi et al, 1993
Gilbert (Arias) syndrome (HBLRG)	#143500	*UGT1A1* (2q37)	Koiwai et al, 1995
Dubin–Johnson syndrome (HBLRDJ)	#237500	*ABCC2* (10q24)	Kajihara et al, 1998
Rotor syndrome (HBLRR)	#237450	*SLCO1B3* (12p12), *SLCO1B1* (12p12)	van de Steeg et al, 2012

Abbreviations: BA, biliary atresia; OMIM, Online Mendelian Inheritance in Man; PFIC, progressive familial intrahepatic cholestasis.

Polymorphism Phenotyping data collection version 2 (PolyPhen-2),[29] and Mutationtaster tool (available from www.mutationtaster.org).[30] Validation of selected variants was done through polymerase chain reaction and direct nucleotide sequencing (Sanger sequencing).

Results

Eleven female and 9 male infants with BA with a median age at operation of 80 days (range: 41–178 days) were included in the study. After a Kasai's operation (hepatic portoenterostomy), 7 cases achieved jaundice clearance, 3 cases were partially improved, and 10 showed no improvement, including a case with rapid progression of ascites leading to respiratory failure and death at postoperative day 22. None of the cases had apparent phenotypes of any congenital syndrome at presentation or during the follow-up period. Two cases had associated anomalies, one case with ventricular septal defects together with pulmonary stenosis (B7) and one case with cleft lip and palate and Meckel's diverticulum (B92). Case B92 later developed a convulsive disorder compatible with Lennox–Gastaut syndrome. Computerized tomography of the brain in this case showed no structural lesion. Serology for toxoplasmosis, other viruses, rubella, cytomegalovirus, and herpes simplex (TORCH)-related infections reported positive cytomegalovirus (CMV) infection in twocases, toxoplasma immunoglobulin G (IgG) in one case, and hepatitis B (HBV) in one case (►**Table 2**). In all cases, histopathologies of the extrahepatic bile ducts and biopsied livers were consistent with BA.

Rare nonsynonymous variants of the studied genes were detected in 11 cases (►**Table 2**). *JAG1* was the most frequent mutated genes, detected in four cases, followed by *MYO5B* in two cases. Except for *NOTCH2*, genes listed in Category I (*RFX6*, *ERCC4*, *KCNH1*, *MLL2*, and *JAG1*) were found to have variants in our BA cases and those variants all had reported frequencies of less than 0.01 in the dbSNP database. Five of the 13 variants had previously been reported to be

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Table 2 Clinical and pathological characteristics of the 11 BA cases with rare variants of the genes studied

Serial no.	Mutated gene	Sex	Initial bilirubin (DB/TB) (mg/dL) and serology	Age[a] (d)	Jaundice clearance	Last follow-up age	Follow-up remarks
B2	*JAG1* *ABCC2*	M	10.57/11.41 CMV-IgG and IgM positive	41	Not improved	6 mo (loss)	Early cirrhosis at 6 mo, lost to follow-up
B6	*UGT1A1*	M	11.92/12.75	77	Jaundice free	13 y	Late portal hypertension and hypersplenism at 10 y, improved after splenic embolization
B7[b]	*JAG1*	F	9.12/9.12	77	Improved	8 y	Late portal hypertension and esophageal varices, underwent transplantation at 9 y
B8	*MYO5B*	M	13.47/19.05 HBsAg positive	149	Not improved	6 mo	Early cirrhosis, died of gram-negative sepsis
B52	*MYO5B*	F	11.25/14.48	120	Jaundice free	8 y	Late portal hypertension and hypersplenism
B67	*JAG1*	F	13.03/13.85	178	–	–	Operative death at postop day 22 due to massive ascites and gram-negative sepsis
B73	*RFX6*	M	13.56/15.38	65	Jaundice free	5 y	Doing well
B80	*MLL2*	F	18.73/19.36 CMV-IgG and IgM positive	84	Not improved	8 mo	Died of respiratory syncytial virus associated pneumonia
B86	*ERCC4*	M	6.21/7.95	54	Not improved	1 y (dead)	Early cirrhosis at 6 mo, lost to follow-up
B95	*KCNH1*, *JAG1*	F	16.29/16.80 Toxoplasma IgG positive	123	Not improved	2 y	Portal hypertension, ascites
B101	*ABCB11*	F	11.43/12.47	142	Not improved	1 y	Early cirrhosis at 6 mo

Abbreviations: BA, biliary atresia; CMV, cytomegalovirus; DB, direct bilirubin; IgG, immunoglobulin G; IgM, immunoglobulin M; TB, total bilirubin.
Note: Age[a]; age at operation date; B7[b], BA with associated congenital cardiac defects.

pathogenic by other studies, 3 of 13 were predicted to be damaging (score < 0.05) by the SIFT tool, 3 of 13 were predicted by PolyPhen-2 to be at least "possibly deleterious" (PolyPhen-2 score > 0.15), and 7 of 13 were predicted to be "disease causing" by the Mutationtaster tool (►**Table 3**). All variants were confirmed by Sanger sequencing.

The clinicopathologies of the 11 cases with genetic variants are shown in ►**Table 2**.

Discussion

Bile stasis, ductular proliferation, and periportal inflammation are mainstay pathological features in BA when giant cell hepatocytes and bridging fibrosis can be found as associated pathological features.[31,32] However, none of the mentioned histopathologies are specific to BA. Bile plug, periportal infiltration of inflammatory cells, and giant cell formation can also be detected in "neonatal hepatitis syndrome," and other obstructive cholangiopathies in infants such as certain types of PFIC[33,34] and BH. The question is raised about whether obstruction of the extrahepatic biliary tree found in BA is not a distinct disease entity, but a shared pathological feature that can be found in a variety of cholangiopathy disorders. Our study followed the trail using a whole exome sequencing approach in several BA cases by focusing on rare variants of those genes that have been reported for their association with infantile nonhemolytic hyperbilirubinemia, from those with evidence in OMIM that BA can be a part of their syndrome in Category I, to Category III in which hyperbilirubinemia is the primary event. The study found that rare nonsynonymous variants within the genes were annotated in half of our cases, most frequently in *JAG1*.

Germline mutations of *JAG1* are the molecular signature of AGS which is associated with BH.[35] Interestingly, there are previous studies reporting mutations of *JAG1* in BA. In an analysis of *JAG1* mutations in 98 sporadic BA cases, Kohsaka et al from Japan found *JAG1* mutations in 9 cases, of which 8 had poor outcome after a hepatic portoenterostomy and ended up with early liver transplantation.[36] None of the cases in that series had any other features of AGS. Of our 4 cases with *JAG* mutations, 3 had no other anomalies suggestive of AGS, and one had cyanotic heart disease. Taken together, the clinical presentation of cardiac anomaly, severe liver deterioration with heterozygous nonsense mutation of *JAG1* in this case, AGS could be diagnosed. However, pathological review of the liver and bile duct clearly showed that this was not a case of "paucity of bile ducts" described in classical BH, but rather an inflammatory cholangiopathy found in BA. The evidence implies that bile duct pathology in AGS may involve the extrahepatic biliary tree. Three of our 4 mutations in JAG1 had either previously been reported in AGS or were predicted to be pathogenic by the SIFT tool. Recent studies using *JAG1* as a molecular marker of AGS have suggested that in early infancy, AGS-associated BH could mimic BA and genetic testing might help differentiate the two conditions.[37,38] Consistent with a report from Japan, all cases with *JAG1* mutations in our study had poor outcome, which suggests that molecular pathology is potentially useful in infants with a diagnosis of BA as the data may prognosticate the result of hepatic portoenterostomy or identify those who need early hepatic transplantation. A large series from Japan where targeted sequencing was performed in 109 infants with non-BA cholestasis reported a relatively high incidence of *JAG1* mutations (13 cases), and most of these (12/13) had a clinical syndrome compatible with AGS.[21] In addition to *JAG1* and *NOTCH2* mutations, mutations of various genes associated with PFIC, including *ABCB11* and *MYO5B*, were detected in our study. The evidence suggests that certain types of PFIC could be present in infancy and their manifestation may mimic BA in the same way that *JAG1* associated BH does.[39]

Genetic mutations found in nonobstructive infantile cholestasis, Crigler–Najjar syndrome (*UGT1A1*), and Dubin–Johnson syndrome (*ABCC2*), were detected in 2 of our 20 cases. As the case harboring *ABCC2* mutation also had a *JAG1* mutation, the contribution of *ABCC2* to the pathogenesis of cholestasis in the case might have been only a coexisting lesion or it is possible that the two lesions together had a synergistic role in the severity of cholestasis. Consistent with this case, another case with concomitant *JAG1* and *KCNH1* mutations had relatively more severity and poor treatment outcome when compared with other cases. As *UGT1A1* is functionally involved in the transporting enzyme uridine 5'-diphospho (UDP)-glucuronyl transferase and a patient with the syndrome usually has unconjugated hyperbilirubinemia, the mutation in *UGT1A1* found in our BA case was not likely a disease-causing mutation. Alteration in transportation-related genes may play the role of a genetic modifier that may affect the severity of the cholestatic disease. Detection of pathogenic mutations previously annotated in clinical syndromes that have BA as a part of their phenotypes suggests that that polysplenia syndrome may not be the only malformation associated with BA. Other syndrome-related genotypes may contribute to the pathogenesis of cholangiopathy although their phenotypes are not apparent.

BA can develop secondary to other etiologies such as infection, immune disorders, or vasculopathy.[40] The main limitation of our study was the small number of cases, owing to the limitation in resources and our requirement for a clear selection of cases with supporting pathology compatible with BA. A lack of parental data precluded any examination of that whether the mutations were inherited or not. In addition, the DNA samples were from unaffected liver tissue, and thus we could not make any clear judgment as to whether the mutations were a somatic or germline change. A recent study hypothesized that genetic changes in BA might be specific to the liver and the biliary tissue and such somatic events can be explained by somatic mosaicism.[40]

In conclusion, our study used whole exome studies to evaluate genetic variants in 20 BA cases, focusing on rare variants in genes previously reported in infantile hyperbilirubinemia. The study found 13 variants from 11 cases. The evidence from our study suggests that BA might be a shared pathology of various conditions that manifest as infantile cholestasis, especially BH and PFIC. Genetic

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Table 3 Rare variants detected in the study in infantile cholestasis-related genes

Sample no.	SNP ID	Gene	Nucleotide change (zygosity)	Amino acid change	Chr: position	MAF (dbSNP)	Effect prediction score		
							SIFT	PolyPhen-2	MUTTASTER
B2	rs200929472	*JAG1*	c.2048G > A (HET)	p.Arg683His	20:10645421	0.0002	0.51 (T)	0.037 (B)	29, 0.99 (D)
	rs370049107	*ABCC2*	c.3379G > A (HET)	p.Val1127Ile	10:99834500	0.0002	1.00 (T)	0.001 (B)	29, 0.60 (P)
B6	rs35350960	*UGT1A1*	c.686C > A (HET)	p.Pro229Gln	2:233760973	0.0030	0.13 (T)	0.662 (P)	76, 0.99 (P)
B7	rs876660980	*JAG1*[a]	c.703G > A (HOM)	p.Arg235ST	20:10656450	< 0.0001	N/A (D)	N/A (D)	N/A, 1.00 (D)
B8	rs757769301	*MYO5B*	c.3254G > A(HET)	p.Arg1085Gln	18:49878967	< 0.0001	0.30 (T)	0.001 (B)	43, 0.96 (D)
B52	rs117920737	*MYO5B*[a]	c.197A > C (HET)	p.Asp66Ala	18:50040256	0.001	0.00 (D)	0.019 (B)	126, 1.00 (D)
B67	rs527420845	*JAG1*	c.2884A > G (HET)	p.Thr962Ala	20:10641492	0.0003	0.00 (D)	0.881 (D)	58, 1.00 (D)
B73	rs775365826	*RFX6*	c.2021C > T (HET)	p.Ala674Val	6:116927162	< 0.0001	0.36 (T)	0.00 (B)	64, 1.00 (P)
B80	rs3782356	*MLL2*[a]	c.15671G > A (HET)	p.Arg5224His	12:4902695	0.002	0.00 (D)	0.096 (B)	29, 1.00 (D)
B86	rs55761944	*ERCC4*[a]	c.241G > T (HET)	p.Val81Phe	16:13922064	0.0003	0.04 (D)	0.939 (D)	50, 1.00 (D)
B95	rs139318016	*KCNH1*	c.2372G > A (HET)	p.Arg791His	1:210683879	0.0004	0.12 (T)	0.977 (D)	98, 1.00 (P)
	rs183974372	*JAG1*[a]	c.133G > T (HET)	p.Val45Leu	20:10672955	0.002	0.29 (T)	0.038 (B)	32, 0.95 (D)
B101	rs372939910	*ABCB11*	c.2135A > G (HET)	p.Leu712Ser	2:168964249	0.0004	0.42 (T)	0.001 (B)	145, 1.00 (P)

Abbreviations: HET, heterozygous; HOM, homozygous; MAF, minor allele frequency in 1,000 Genomes Project Phase 3; MUTTASTER, Mutation Taster–AA change score and probability value (D, disease causing; P, polymorphism); N/A, not applicable; PolyPhen, Polymorphism Phenotyping data collection (B, benign; P, possibly deleterious; D, deleterious); SIFT, scale-invariant feature transform (T, tolerated; D, damaging); SNP, single-nucleotide polymorphism.

Note: [a]The same position with prior reported to be pathogenic in other studies.

studies may help differentiate these conditions and direct appropriate management.

Conflict of Interest
None.

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