Defects in Myosin VB Are Associated With a Spectrum of Previously Undiagnosed Low γ-Glutamyltransferase Cholestasis

Yi-Ling Qiu,1 Jing-Yu Gong,2 Jia-Yan Feng,3 Ren-Xue Wang,4 Jun Han,5 Teng Liu,2 Yi Lu,1 Li-Ting Li,1 Mei-Hong Zhang,2 Jonathan A. Sheps,4 Neng-Li Wang,2 Yan-Yan Yan,2 Jia-Qi Li,2 Lian Chen,3 Christoph H. Borchers,5 Bence Sipos,6 A.S. Knisely,7 Victor Ling,4 Qing-He Xing,8 and Jian-She Wang2,9

Hereditary cholestasis in childhood and infancy with normal serum gamma-glutamyltransferase (GGT) activity is linked to several genes. Many patients, however, remain genetically undiagnosed. Defects in myosin VB (MYO5B; encoded by MYO5B) cause microvillus inclusion disease (MVID; MIM251850) with recurrent watery diarrhea. Cholestasis, reported as an atypical presentation in MVID, has been considered a side effect of parenteral alimentation. Here, however, we report on 10 patients who experienced cholestasis associated with biallelic, or suspected biallelic, mutations in MYO5B and who had neither recurrent diarrhea nor received parenteral alimentation. Seven of them are from two study cohorts, together comprising 31 undiagnosed low-GGT cholestasis patients; 3 are sporadic. Cholestasis in 2 patients was progressive, in 3 recurrent, in 2 transient, and in 3 uncategorized because of insufficient follow-up. Liver biopsy specimens revealed giant-cell change of hepatocytes and intralobular cholestasis with abnormal distribution of bile salt export pump (BSEP) at canaliculi, as well as coarse granular dislocation of MYO5B. Mass spectrometry of plasma demonstrated increased total bile acids, primary bile acids, and conjugated bile acids, with decreased free bile acids, similar to changes in BSEP-deficient patients. Literature review revealed that patients with biallelic mutations predicted to eliminate MYO5B expression were more frequent in typical MVID than in isolated-cholestasis patients (11 of 38 vs. 0 of 13).

Conclusion: MYO5B deficiency may underlie 20% of previously undiagnosed low-GGT cholestasis. MYO5B deficiency appears to impair targeting of BSEP to the canalicular membrane with hampered bile acid excretion, resulting in a spectrum of cholestasis without diarrhea. (HEPATOLOGY 2017;65:1655-1669).

Hereditary cholestasis with conjugated hyperbilirubinemia in children presents as a range of disorders, from transient neonatal cholestasis (TNC) through benign recurrent intrahepatic cholestasis (BRIC) to progressive familial intrahepatic cholestasis (PFIC). Of children with such cholestasis and low or normal serum gamma-glutamyltransferase (GGT) activity (GGT <100 IU/L; “low-GGT cholestasis”), two thirds carry either ATP8B1 or ABCB11 mutations; the remaining instances of childhood cholestasis with conjugated...
hyperbilirubinemia and low GGT can sometimes be attributed to mutations in TJP2, which encodes tight junction protein 2 (TJP2); in genes involved in bile acid synthesis, including HSD3B7, AKR1D1, CYP7B1, AMACR, and CYP27A1; in VPS33B and VIPAS39, which cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome; and in NR1H4, encoding the nuclear farnesoid X receptor (FXR) (see Supporting Table S1). However, approximately one fifth of such patients remain without an identified genetic defect, suggesting that mutations at additional loci are responsible for childhood low-GGT cholestasis. Myosin VB (MYO5B), associated with plasma membrane recycling and transcytosis, is essential to polarization of hepatocytes, enterocytes, and respiratory epithelial cells through protein–protein interactions with RAS-related GTP-binding protein 11A (RAB11A), RAB8A, and cystic fibrosis transmembrane conductance regulator, respectively. The interaction of MYO5B with RAB11A also appears essential for targeting bile salt export pump (BSEP) to the canalicular membrane.

Functional deficiency in MYO5B, or absolute deficiency of MYO5B, results in aberrant cell polarity and is the major cause of microvillus inclusion disease (MVID; MIM 251850), an autosomal-recessive disorder causing persistent watery diarrhea manifest in infancy that requires parenteral alimentation and even intestinal transplantation. Cholestasis with normal-range GGT and intractable pruritus has been reported as an atypical symptom or as a side effect of parenteral nutrition in some MVID patients before or after intestinal transplantation and is associated with altered targeting of BSEP to the canalicular membranes of hepatocytes.

To identify the underlying causes of cholestasis of undetermined etiology in patients with normal serum GGT and without demonstrable mutations in known candidate genes, we performed whole-exome sequencing (WES) and targeted sequencing. In a subset of these patients, we identified biallelic mutations in MYO5B, which encodes MYO5B. Through immunostaining and bile acid profiling, we demonstrated that these patients had impaired MYO5B and BSEP expression as well as plasma bile acid profiles similar to those observed in severe primary BSEP/ABCB11 disease (PFIC2). Our work implicates MYO5B mutation as capable of reproducing the full clinical spectrum of isolated low-GGT cholestasis.

**Subjects and Methods**

**SUBJECTS**

Study subjects were Han Chinese enrolled from 2011 to 2016, with informed consent, under a clinical-diagnosis protocol approved by Children’s Hospital and Jinshan Hospital of Fudan University (Shanghai, China) and according to the ethical guidelines of the 1975 Declaration of Helsinki. The following enrollment criteria were used: elevated serum total bilirubin (TB) and direct bilirubin (DB); GGT < 100 IU/L; failure to ascertain an etiology of disease through testing listed in Supporting Table S2; and parental DNA available. All patients

**ARTICLE INFORMATION:**

From the 1The Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, Shanghai, China; 2Department of Pediatrics, Jinshan Hospital of Fudan University, Shanghai, China; 3Department of Pathology, Children's Hospital of Fudan University, Shanghai, China; 4BC Cancer Agency, Vancouver, British Columbia, Canada; 5University of Victoria – Genome BC Proteomics Centre, University of Victoria, Victoria, British Columbia, Canada; 6Institute of General Pathology and Neuropathology, Tübingen University Hospital, Tübingen, Germany; 7Institute of Pathology, Graz Medical University, Graz, Austria; 8Institutes of Biomedical Sciences of Fudan University, Shanghai, China; 9Department of Infectious Diseases, Children's Hospital of Fudan University, Shanghai, China.

**ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:**

Jian-She Wang, M.D., Ph.D.
Department of Pediatrics, Jinshan Hospital of Fudan University
No. 1508 Longhang Road
Jinshan District
Shanghai 201508, China
E-mail: jshwang@shmu.edu.cn
Tel: +86 21 57039426
Qing-He Xing, Ph.D.
Institutes of Biomedical Sciences of Fudan University
Mingdao Building
No. 131 Dongan Road
Xuhui District
Shanghai 200032, China
E-mail: xingqinghe@hotmail.com
Tel: +86 13166095596
Victor Ling, Ph.D.
BC Cancer Agency
675 west 10th Avenue, Room 9-302,
Vancouver, British Columbia, Canada, V5Z 1L3
E-mail: vling@bccrc.ca
Tel: +1-604-675-8101.
were analyzed either by WES or targeted sequencing. The first cohort included 24 patients enrolled from 2011 to 2014. After the identification of 5 cases with MYO5B defects in the first cohort (patients P1-5), we retrospectively reviewed undiagnosed cholestasis patients admitted from 2011 to 2015. From them, we selected 7 more patients with available liver biopsy specimens as a second cohort. Two patients with MYO5B defects were identified in this cohort (P6 and P7) by immunohistochemical (IHC) and DNA sequencing analyses (detailed in Results, Figs. 1–3). Three sporadic instances of MYO5B defects also were identified. Patients 8 and 9 were found using a new genetic screening panel that included MYO5B. Patient 10 was found by WES as part of clinical investigation of recurring cholestasis. None of the patients’ families were related to any other patients’ family.

Twenty-six patients (all Han Chinese) with unexplained high-GGT cholestasis or other forms of liver disease from the same geographical regions were listed as “other-liver-disease controls,” and 338 patients with neurological disorders or unknown genetic disorders without liver disease were used as “nonliver controls.” All controls were analyzed by WES.

GENETIC ANALYSES

Genomic DNA (gDNA) was extracted from ethylenediaminetetraacetic acid (EDTA)-treated peripheral blood cells (QIAamp DNA Blood Mini Kit, Catalog No. 51106; Qiagen, Germany) of the enrolled patients and their available family members. WES was performed using patient gDNA with a SureSelectXT Reagent kit (Catalog No. G9611A; Agilent, Santa Clara, CA, USA), SureSelectXT Human All Exon V5 (Catalog No.5190-6208; Agilent), TruSeq PE Cluster Kit v3-cBot-HS (Catalog No. PE-401-3001; Illumina, San Diego, CA, USA), and HiSeq SBS Kit V4 (Catalog No. FC-401-4003; Illumina). Quantification was performed with an Agilent 2100 Bioanalyzer (Catalog No.G2938A; Agilent), and multiplexed sequencing was done on HiSeq 2500 sequencers with 2 × 150 paired-end modules (Illumina). Total sequencing depth was 100×. WES and annotation were done by Genesky Biotechnologies (Shanghai, China). Supporting Fig. S1 shows the filtering procedures for the WES data. Online resources GeneCards, Orphanet, JuniorDoc online database, ClinVar, OMIM, and PubMed were used to search for genes known to be

FIG. 1. Histological findings in MYO5B mutant patients (original magnification, all images, ×400). On H&E staining, hepatocellular and canalicular cholestasis, lobular disarray, mild inflammation, and portal-tract fibrosis were apparent in all specimens. Giant-cell formation was observed in all patients, with ballooning degeneration of hepatocytes in P4 and P7. CK7 and CK19 immunostaining revealed ductular reaction in all patients but P6, as well as weak heterotopic CK7 expression in some hepatocytes.
pathogenically mutated in liver disease and genes expressed in or functionally related to liver. HGMD, dbSNP, Exome Variant Server (ESP6500), 1000 Genomes, and ExAC Browser were applied to filter common variants. Polyphen2, SIFT, and MutationTaster\(^{(34)}\) were used to predict the pathogenicity of candidate variants. In addition, an internal database (data not shown) was used to filter common variants or common sequencing errors. We also included a candidate list containing reported hereditary disorders with liver presentations or associated with liver metabolism (data not shown). Predicted pathogenic variants of suspect genes

FIG. 2. MYO5B expression in MYO5B mutant patients (original magnification, all principal images, ×200; insets, ×400). (A) Choledochal cyst control without cholestasis; (B) incidentally resected normal liver control (adjoining excised tumor); (C) biliary atresia control with cholestasis. MYO5B Patients P3-P5, P6, and P7: Much coarsely granular pigment was observed in every patient specimen (Fig. 3, P3-P5, P6, and P7), whereas fewer and finer MYO5B-positive granular deposits were observed in the control individuals, mainly distributed around portal areas (Fig. 3 A,B). The size and number of positive granules in the biliary atresia patient (Fig. 3C) were intermediate between those of the patient group and the control group; the granules in this patient were periportal.
were verified through PCR (2*Master Mix, Catalog No. KT201; Tiangen, Shanghai, China) followed by Sanger sequencing in the patient and his or her family members. Low-coverage (coverage lower than 5) exons in candidate genes were also subjected to Sanger sequencing. Primers and PCR conditions are available on request.

**HISTOLOGICAL AND IHC STUDIES**

Specimens of liver were fixed in 4% acetic formalin, embedded in paraffin, cut into 4-micron sections, stained with hematoxylin and eosin (H&E), and immunostained with antibodies against cytokeratin (CK) 7 (monoclonal mouse anti-human, OV-TL12/30, ready to use; Agilent, Santa Clara, CA, USA) and CK19 (monoclonal mouse anti-human, RCK108, ready to use; Agilent). Immunostaining with anti-MYO5B antibodies (N-term human MYO5B, Ab190096; Abcam, Cambridge, UK) and anti-BSEP monoclonal antibody (mouse anti-human, F-6, sc-74500; Santa Cruz Biotechnology, Dallas, TX, USA) were also performed. Specimens from patients and normal controls were stained together on the same slide when performing immunostaining.

For a diagnosis-blinded review of BSEP (ABCB11) immunostaining, unstained slides were sent to B.S. Slides were subjected to heat-induced antigen retrieval (CC1; Ventana Medical Systems, Tucson, AZ) and immunostained with rabbit polyclonal anti-BSEP antibody (HPA 19035, 1:2,000 dilution; Sigma-Aldrich, Taufkirchen, Germany), with diaminobenzidine as chromogen and hematoxylin as counterstain, using a Benchmark Ultra Immunostainer (Ventana). Normal human liver was used as a positive control. Slides were reviewed by two independent pathologists (A.S.K. and B.S.) with no knowledge of associated clinical or genetic information.

**ANALYSIS OF BILE ACIDS IN PLASMA**

Plasma samples were collected from EDTA-treated peripheral blood by centrifugation. Analysis of bile acids in plasma by ultrahigh performance liquid chromatography–electrospray ionization/multiple reaction monitoring mass spectrometry (UPLC–ESI/MRM-MS) with negative ion detection was performed at the University of Victoria–Genome British Columbia Proteomics Centre (Victoria, BC, Canada), using described sample preparation and quantitation procedures.(35)

**GENOTYPE-PHENOTYPE CORRELATION IN MYO5B-MUTATED PATIENTS**

To explore the phenotype-genotype relationship, patients with genetically confirmed MYO5B disease
identified in this study, and those in published reports,\(^{(24,25,27,29,36-38)}\) were divided into isolated cholestasis or MVID subgroups. All mutations were categorized in two ways, severe versus nonsevere, and MYO5B-RAB11A interaction domain-related versus unrelated. Frameshift, nonsense, and classical splicing mutations were defined as severe mutations. Mutations located in the MYO5B-RAB11A interaction domain, or mutations predicted to influence interactions at that domain,\(^{(39)}\) were termed MYO5B-RAB11A domain-related mutations. The proportions of patients with biallelic severe mutations, or with biallelic mutations related to MYO5B-RAB11A interaction, were compared between the subgroups.

**STATISTICAL ANALYSIS**

Fisher’s exact test was performed, using the software package STATA 10 (StataCorp LP, College Station, TX, USA) for mutation frequency analysis, to compare patients carrying biallelic \(MYO5B\) mutations in the first cohort with “other liver-disease controls” and “nonliver disease controls.” The same methods were used to explore the phenotype-genotype relationship in genetically confirmed \(MYO5B\)-mutated patients. To avoid dependence between samples, 1 patient from multipatient sibships was randomly selected for statistical analysis. Rank-sum tests were performed using the software package SPSS 19 (IBM, Armonk, NY) to compare bile acid profiles of \(MYO5B\)-mutated patients with those of PFIC2 patients and controls.

**Results**

**BIALLELIC \(MYO5B\) MUTATIONS ARE ASSOCIATED WITH LOW-GGT CHOLESTASIS WITHOUT RECURRENT DIARRHEA**

We identified 15 \(MYO5B\) mutations, three known and 12 novel, in 10 cholestatic patients. Among these, two nonsense mutations (c.1021C>T, p.Q341X; c.3046C>T, p. R1016X) had been reported in MVID patients without cholestasis,\(^{(27,36)}\) and one missense mutation (c.1604G>A, p.S535N) has a reported frequency of 0.001 in East Asian populations in the ExAC database (http://exac.broadinstitute.org/variant/18-47480747-C-T), but is predicted to be disease causing (Table 1). SIFT and Polyphen2 and/or MutationTaster predicted that the novel mutations would result in loss of MYO5B function or cause disease (Table 1).

In the first cohort of 24 patients, biallelic \(MYO5B\) mutations were detected in 5 patients (P1–P5; Table 1) from four unrelated families. No disease-causing mutation that matched this inheritance mode was detected in other genes associated with cholestasis (Supporting Table S3). In addition, monoallelic \(MYO5B\) mutations were detected in 2 patients (P11 and P12; Table 1). The frequency of \(MYO5B\) mutations is significantly higher in this group than in the “other-liver-disease controls” (5 of 24 vs. 0 of 26, Fisher’s exact test; \(P = 0.02\)) and in the “nonliver controls” (one sample in this group contained two \(MYO5B\) variants and was treated as a compound heterozygote, though not confirmed as such because of unavailability of parental samples; 5 of 24 vs. 1 of 338, Fisher’s exact test; \(P = 4.84 \times 10^{-6}\)), indicating a strong association between \(MYO5B\) mutation and low-GGT cholestasis without diarrhea.

In the second cohort, two \(MYO5B\) mutations were found in 2 of the 3 patients sent for targeted sequencing because liver biopsy had found histopathologic features like those of \(MYO5B\) disease as established in the first cohort (Figs. 1–3). However, compound heterozygosity was not confirmed given that parental samples were not available. No \(MYO5B\) mutation was found in either the third patient who underwent targeted sequencing or the remaining 4 who underwent WES.

Two mutations in \(MYO5B\) were found in patient P8 after introduction of a new screening panel that included \(MYO5B\) (Table 1). Compound heterozygosity was confirmed by studies in her parents. Her younger brother, with icterus (P9), harbored the same mutations. Homozygous \(MYO5B\) mutation was discovered by WES in P10, who had recurrent bouts of cholestasis.

**CLINICAL FEATURES OF CHOLESTATIC PATIENTS WITH \(MYO5B\) MUTATIONS**

All 10 patients with two \(MYO5B\) mutations were born at term, with normal weight, to healthy, unrelated parents. Pregnancy and parturition were unremarkable in all mothers. No patient suffered recurrent diarrhea or received parenteral alimentation. All presented with cholestasis and elevated DB, low GGT, mildly elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values, and elevated serum total bile acid (TBA) concentrations. Blood glucose, ammonia, and alpha-fetoprotein (AFP) values were all within...
expected ranges. The rest of their examination results were unremarkable. The principal medications routinely administered were ursodeoxycholic acid (UDCA) and fat-soluble vitamins; cholestyramine was added to alleviate unresolved pruritus and/or cholestasis. Other major clinical and biochemical details are shown in Table 2.

P1 and P3 presented with persistent cholestasis. P1 received routine UDCA therapy for 1 month; his parents substituted Traditional Chinese Medicine for UDCA without any improvement. He was lost to follow-up for around 2 years. When seen again, aged 6 years, he had mild jaundice and pruritus (TB, 85.7 umol/L; DB, 48.7 umol/L; ALT, 70 IU/L; AST, 80 IU/L). He was returned to cholestyramine and fat-soluble vitamin therapy, with substantial improvement in symptoms (Table 2), but without catch-up in growth (height, 106 cm, <3rd percentile, August 2016). P3 seemed nonresponsive to routine treatment, was listed for liver transplantation (LT) elsewhere at age 2.3 years, and died untransplanted 4 months later.

P5, P8, and P10 suffered from intermittent (recurrent) cholestasis. Each had two episodes of cholestasis, with pruritus during bouts, and was asymptomatic between episodes. For P5, the first episode began at age 6 months. No trigger was recognized. Treatment was given elsewhere (details not available), and cholestasis

### Table 1. Mutations in MYO5B (NM_001080467) in Low-GGT Cholestasis Patients in This Study*

| Patient | Mutation | Predicted Effects | MYO5B Domain | Zygosity | Origin | HGMD ID | Predicted Effect, MYO5B-Rab11a Domain | SIFT | Polyphen |
|---------|----------|-------------------|--------------|----------|--------|---------|--------------------------------------|------|----------|
| **Patients with biallelic mutation** | | | | | | | | | |
| P1 and P2 | c.3538-1G>A | Splicing | Coiled coil | Heterozygous | Father | — | Abolish interaction | N/A | N/A |
| P3 | c.2414+5G>T | Splicing | IQ | Heterozygous | Mother | — | None | N/A | N/A |
| | c.1201C>T | p.R401C | Head | Heterozygous | Father | — | None | Deleterious | Possibly damaging | N/A |
| | c.1021C>T | p.Q341X | Head | Heterozygous | Mother | CM108966 | Abolish interaction | N/A | N/A |
| P4 | c.3237G>C | p.Q1079H | Coiled coil | Heterozygous | Father | — | None | Deleterious | Possibly damaging | P3 |
| | c.1604G>A | p.S535N | Head | Heterozygous | Mother | — | Abolish interaction | Tolerated | Possibly damaging | P3 |
| P5 | c.796T>C | p.C266R | Head | Heterozygous | Father | — | Abolish interaction | Deleterious | Probably damaging | P3 |
| P6† | c.1748G>A | p.S583N | Head | Heterozygous | ND | — | Abolish interaction | Deleterious | Possibly damaging | P3 |
| | c.2801T>G | p.I934S | Coiled coil | Heterozygous | ND | — | Abolish interaction | Deleterious | Possibly damaging | P3 |
| | c.2090_2090delG | p.R697Gfs*74 | Head | Heterozygous | ND | — | Abolish interaction | N/A | N/A |
| P7† | c.4852+11A>G | Splicing | Tail | Heterozygous | ND | — | N/A | N/A | N/A |
| | c.3046C>T | p.R1016X | Coiled coil | Heterozygous | Mother | CM085576 | Abolish interaction | N/A | N/A |
| P8 and P9 | c.437C>T | p.S158F | Head | Heterozygous | Father | — | None | Deleterious | Possibly damaging | P3 |
| | c.2470C>T† | p.R824C | IQ | Heterozygous | Father | — | None | Deleterious | Possibly damaging | P3 |

| **Patients with monoallelic mutation** | | | | | | | | | |
| P11 | c.2470C>T† | p.R824C | IQ | Heterozygous | Mother | — | None | Deleterious | Possibly damaging | P3 |
| P12 | c.1136G>C† | p.R379P | Head | Heterozygous | Father | — | None | Deleterious | Possibly damaging | P3 |

*MutationTaster assessed all mutations as disease-causing.
†While this article was under review, two additional MYO5B mutations causing isolated cholestasis were reported. One is c.2470C>T, p.R824C. The other is 1135C>T, p.R379C, which changes the same codon as 1136G>C, reported here.

Abbreviations: N/A, not applicable; IQ, Isoleucine-glutamine (IQ) calmodulin-binding consensus sequence; ND, not done.
| Patients | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|----------|----|----|----|----|----|----|----|----|----|-----|
| Sex | M | M | F | M | M | M | F | M | M | M |
| Maternal ICP | — | — | — | — | — | — | — | — | — | — |
| Maternal spontaneous abortion | 0 | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Siblings | One affected brother (P2) | One affected brother (P1) | One healthy brother | None | None | One healthy brother | None | One affected brother (P9) | One affected sister (P8) | One healthy sister |
| Presenting age | 8mo | 19mo | 1mo | 2d | 6mo | 15d | 3mo | 7mo | 1mo | 7mo |
| Status at first assessment | — | — | — | — | — | — | — | — | — | — |
| Age | 9mo | 18mo | 2mo | 1mo | 6mo | 3mo | 4mo | 10.0y | 6mo | 11mo |
| Lithiasis | — | — | — | — | — | — | — | — | — | — |
| Hepatomegaly | + | + | + | + | + | + | — | — | + | + |
| Splenomegaly | — | — | — | — | — | — | — | — | — | — |
| Pruritus | + | + | + | — | + | — | — | + | — | — |
| TB | 207.9 | 133.5 | 133 | 158 | 205.7 | 206.7 | 117.2 | 222.9 | 39.2 | 300.6 |
| DB | 135.9 | 90.8 | 47.6 | 100.5 | 137.1 | 146.5 | 58 | 120.7 | 29 | 229 |
| ALT | 36 | 40 | 57 | 255 | 88 | 84 | 148 | 24 | 62 | 33 |
| AST | 49 | 41 | 48 | 434 | 88 | 196 | 352 | 35 | 55 | 62 |
| ALP | 524 | 688 | NA | 342 | 1,010 | 1,364 | 888 | 452 | 1,062 | 649 |
| GGT | 14 | 17 | 42 | 47 | 10 | 99 | 85 | 13 | 10 | 9 |
| TP | 55.4 | 57 | NA | 50.1 | 97.1 | 68 | NA | 59.3 | 55.2 | 52 |
| Alb | 27.3 | 32.6 | NA | 33.4 | 34.5 | 38.3 | 24.6 | 41 | 38 | 31.6 |
| TBA | NA | 366.9 | NA | 114 | 240.9 | 180.2 | 21.2 | 222.4 | 206.2 | 461.4 |
| Liver biopsy age | — | — | 9mo | 1mo | 2y | 2.5mo | 4mo | — | — | — |
| Status at most recent assessment | — | — | — | — | — | — | — | — | — | — |
| Age | 7.4y | 4.4y | 2.3y | 2.5y | 6.9y | 7mo | 6.5mo | 10.4y | 8mo | 12mo |
| Height (cm)(40,41) | 106 (<3%) | 117 (97%) | <80 (<3%) | 95 (90%) | 106 (<3%) | 60 (<3%) | NA | 137 (50%) | NA | 78 (75%) |
| Weight (kg)(40,41) | 16.5 (<3%) | 19 (70%) | 9.5 (<3%) | 131 (50%) | 16 (<3%) | 6 (<3%) | 5.5 (<3%) | 28 (25%) | NA | 10 (50%) |
| TB | 32.4 | 6.9 | 133.5 | 9.7 | 12.4 | 118.8 | 194.9 | 237.1 | 46.8 | 173.1 |
| DB | 13.9 | 1.5 | 113.9 | 1.9 | 6.2 | 82.2 | 106.4 | 124.2 | 26.6 | 132.1 |
| ALT | 85 | 42 | 76 | 16 | 33 | 88 | 94 | 37 | 61 | 24 |
| AST | 82 | 39 | 64 | 31 | 28 | 172 | 193 | 24 | 53 | 37 |
| ALP | 1,721 | 295 | 687 | 272 | 402 | 700 | 711 | 341 | 1,489 | 257 |
| GGT | 21 | 15 | 12 | 15 | 9 | 89 | 42 | 18 | 10 | 19 |
| TP | 71 | 68 | 61.1 | 71 | 65.6 | 56.7 | 62.6 | 71 | 65 | 60.2 |
resolved after 6 months, but recurred after a fever at age 6 years. This second episode lasted for 7 months and was treated routinely with cholestyramine. With resolution of cholestasis, all biochemical test results for P5 returned to normal values. Of interest is that P5 was diagnosed with sensorineural deafness from age <1 year and cholecystolithiasis at age 4 years. For P8, the first episode began at age 7 months. No trigger was recognized. Traditional Chinese Medicine was given; details are not available. Cholestasis resolved after 6 months and recurred, with pale stools, after taking cefixime and amoxicillin at age 10 years (March 2016), without menarche. Routine treatment was given; her parents replaced these with some folk prescription 1 month later. This bout has not resolved to date. Of interest in P8 is a history of loose stools, but not of watery diarrhea, until aged 3 years. Of interest in P10 is that both bouts of cholestasis (at 7 and 11 months) were preceded by diarrhea for 10 days that resolved before pruritus and icterus began. No trigger was recognized. Details of previous treatments elsewhere were unavailable. P10 seemed to respond to UDCA therapy in the second episode, with alleviated jaundice and normalizing clinical-laboratory test results before discharge.

P2 and P4 had single bouts of cholestasis that resolved (transient rather than recurrent cholestasis, at least to date), without recognized triggers. P2 only received UDCA for less than 1 week, switching to methylprednisolone at his parents’ discretion. Interestingly, P2, differed from his elder brother P1, carrying the same MYO5B mutations, in showing normalized serum bilirubin and other liver function tests 3 weeks later (age 20 months) through to last follow-up in January 2016 (age 4.4 years; Table 2). P4 had onset of cholestasis from 2 days old with hepatosplenomegaly (liver 3 cm below right costal arch and spleen 2 cm below left costal arch), but, after nearly 1 year of routine UDCA therapy with cholestyramine, he recovered with normal height and weight and mild hepatomegaly (2 cm below right costal arch; see Supporting Fig. S2 for laboratory values and management).

P6, P7, and P9 could not be classified as transient or recurrent cholestasis because of insufficient clinical follow-up.

### HISTOPATHOLOGICAL AND IMMUNOHISTOPATHOLOGICAL FINDINGS

Core needle biopsy specimens of liver were available for P3, P5, P6, and P7. A wedge biopsy specimen of liver was available for P4. Liver controls from children

| Patients | Characterization | Alb (g/L) | TBA (umol/L) | Outcome |
|----------|------------------|----------|--------------|---------|
| P1       | Persistent cholestasis | 42       | 39.4         | Mild cholestasis |
| P2       | Transient cholestasis | 3.9       | 48.4         | Recovered |
| P3       | Transient cholestasis | 4.5       | 43.8         | Recovered |
| P4       | Transient cholestasis | 47       | 41.8         | Recovered |
| P5       | Persistent cholestasis | 47       | 41.1         | Insufficient follow-up |
| P6       | Persistent cholestasis | 43.6     | 294.9        | Insufficient follow-up |
| P7       | Persistent cholestasis | 41.8     | 209.1        | Insufficient follow-up |
| P8       | Persistent cholestasis | 42.6     | 41.1         | Insufficient follow-up |
| P9       | Persistent cholestasis | 43       | 21.9         | Insufficient follow-up |
| P10      | Insufficient follow-up | 41       | 41           | Insufficient follow-up |

Abbreviations and (expected ranges), biochemical tests: TB, total bilirubin (5.0-17.1 umol/L); DB, direct bilirubin (0-1.7 umol/L); ALT, alanine transaminase (0-40 IU/L); AST, aspartate aminotransferase (0-40 IU/L); ALP, alkaline phosphatase (35-55 g/L); TBA, total bile acids (0-10 umol/L).
without cholestasis were a wedge biopsy specimen obtained at choledochal cyst excision (A) and non-neoplastic liver incidentally resected at hepatoblastoma excision (B). Wedge biopsy specimens of liver taken at hepatic portoenterostomy in extrahepatic biliary atresia served as liver controls from children with cholestasis (control A in Fig. 1; controls A, B, and C in Figs. 2 and 3). Subsequent evaluation (B.S., A.S.K.) included material from P3, P4, and P5 as well as from a wedge biopsy specimen of liver from an infant with known ABCB11 mutation and predicted BSEP deficiency (NM_003742: c.1243C>T, p. I498T / c.1493T>C, R415X, Fig. 3D) and discarded tissue from a healthy liver donor (Fig. 3E). Hepatocellular and canalicular cholestasis, with lobular disarray and giant-cell change of hepatocytes, was observed in all patient specimens (Fig. 1). Marking for CK7 and CK19 highlighted slight ductular reaction in all patient specimens other than that from P6. Marking for MYO5B, in the form of fine granules, was scant in control noncholestatic liver; it was observed principally in periportal regions (Fig. 2, controls A and B). Granules were larger and more numerous in control cholestatic liver (Fig. 2C), but were similarly distributed. Coarsely granular marking for MYO5B was diffusely present in all MYO5B patient specimens. Marking for BSEP at bile canaliculi was initially assessed as decreased in P3-P7, with displacement into cytoplasm in P5. Separate immunostaining with patient-blinded review of P3-P5 and controls A-E (B.S., A.S.K.) found no expression of BSEP in P3 or in the patient with a known ABCB11 mutation. In both P4 and P5, BSEP marking at bile canaliculi was assessed as weak (Fig. 3). ABCB4 immunostaining showed disorganized canalicular

FIG. 4. Bile acids in plasma of MYO5B mutant patients. Bile acid (BA) profiles obtained by UPLC–ESI/MDM-MS (μM) of plasma from 4 patients carrying MYO5B defects, compared to those from 16 patients confirmed to harbor biallelic ABCB11 mutations and 20 healthy controls. In the MYO5B group, P1, P2, and P4 refer to patients 1, 2, and 4, respectively, and (P5-1) and (P5-2) both refer to patient 5, sampled twice, during and after a bout of cholestasis. (A) TBAs based on 62 standards that cover all major bile acids and many rare bile acids (see Supporting Table S4); (B) total conjugated bile acids, including glycol-CDCA, glyco-CA, tauro-CDCA, and tauro-CDCA; (C) total free bile acids, including CA, CDCA, DCA and LCA; (D) total primary bile acids, including CA, CDCA, glyco-CA, glycol-CDCA, tauro-CA, and tauro-CDCA; (E) total secondary bile acids, including DCA, LCA, 7-KLCA, 12-KLCA, 67-diKLCA, and DioxyLCA; (F) total UDCA including free, glycol-, and tauro-UDCA and their sulfated forms. The number below each of the MYO5B and ABCB11 groups is the P value of the group versus controls.
marking in MYO5B patients whereas crisply well-defined canalicular distribution was observed in normal controls (Supporting Fig. S3).

**PLASMA BILE ACID PROFILES**

Plasma bile acid profiles during cholestasis in P1, P2, P4, and P5 were compared with those of 16 patients with cholestasis genetically confirmed as associated with ABCB11 mutation and those of 20 healthy donors. A significant increase in plasma concentrations of bile acids over healthy controls was observed in the 4 MYO5B-mutated patients, with much higher values for total, primary, and conjugated bile acids as well as for UDCA (Fig. 4). The bile acid profiles in MYO5B mutation were very similar to those in ABCB11 mutation (Fig. 4). This suggests stagnation of hepatocellular secretion of these bile acid species into bile in both disorders. The increase of total bile acids in plasma likely resulted from both the administration of UDCA (<200 μM) and primary cholestasis (accounting for up to 400 μM of total serum bile acids; cf. Fig. 4A,F). Concentrations of free bile acids in plasma were significantly lower for MYO5B-mutated patients than for healthy controls (Fig. 4C), consistent with poor biliary secretion of bile acids and decreased concentrations of bile acids in chyme. Of note is that P2 and P4, in whom cholestasis resolved after treatment with UDCA and other medications, had higher concentrations of TBAs, secondary conjugated bile acids, and UDCA in plasma than did P1 and P5, who had persistent cholestasis (Fig. 4A,B,D,E,F). Bile acid profiles in plasma from P2 obtained before and after resolution of cholestasis were also compared (Table 3). TBA concentrations fell 100-fold with resolution. Of interest is that whereas glycine conjugates of bile acids typically predominate in humans, taurine-conjugated bile acid concentrations rose in P2 after resolution of cholestasis (Supporting Table S4). The significance of these differences is unknown.

**TABLE 3. Plasma Bile Acid Profiles, Patient 2 While Jaundiced and When Recovered; Median-Value Profiles, Plasma of Patients With ABCB11 Disease Manifest as Nonremitting Cholestasis (n = 16), of Patients With Cholestasis of Unknown Etiology (n = 19), and of Healthy Controls (n = 20)**

| Bile Acid (μM) | Icteric | Recovered | ABCB11 Disease | Cholestasis of Unknown Etiology | Healthy Children |
|---------------|---------|-----------|----------------|-----------------------------|-----------------|
| Cholic acid   | 0.0193  | 0.0485    | 0.0185         | 0.0208                      | 0.0449          |
| Deoxycholic acid | 0.0317 | 0.1130    | 0.0417         | 0.0376                      | 0.1181          |
| Lithocholic acid | 0.0008 | 0.0457    | 0.0013         | 0.0008                      | 0.0020          |
| Allocholic acid | 0.0044 | 0.0054    | 0.0008         | 0.0026                      | 0.0072          |
| Chenodeoxycholic acid | 0.0158 | 0.0747    | 0.0280         | 0.0368                      | 0.1582          |
| Dehydrocholic acid | 0.0021 | 0.0563    | 0.0050         | 0.0093                      | 0.0093          |
| Ursodeoxycholic acid | 0.0266 | 0.0389    | 0.1224         | 0.0502                      | 0.1212          |
| Nordoxycholic acid | 0.0004 | 0.0015    | 0.0010         | 0.0008                      | 0.0007          |
| α-Muricholic acid | 0.0030 | 0.0295    | 0.0018         | 0.0058                      | 0.0135          |
| β-Muricholic acid | 0.0003 | 0.0088    | 0.0009         | 0.0007                      | 0.0049          |
| Glycochenodeoxycholic acid | 72.2260 | 0.7936   | 70.8114        | 41.8743                     | 2.0658          |
| Glycocholic acid | 26.5625 | 0.2741    | 30.6065        | 9.6260                      | 0.4612          |
| Glycodeoxycholic acid | 0.0674 | 0.0542    | 0.0272         | 0.0145                      | 0.0624          |
| Glycohyodeoxycholic acid | 0.0000 | 0.1047    | 0.0000         | 0.0000                      | 0.0000          |
| Glycoursodeoxycholic acid | 30.0986 | 0.1922   | 28.0995        | 16.9341                     | 0.2608          |
| Glycolithocholic acid | 0.0131 | 0.0032    | 0.0192         | 0.0081                      | 0.0026          |
| Glycohyocholic acid | 0.9492 | 0.0270    | 0.5494         | 0.6265                      | 0.0429          |
| Taurodeoxycholic acid | 0.0991 | 0.0231    | 0.0151         | 0.0149                      | 0.0219          |
| Taurochenodeoxycholic acid | 67.7097 | 0.5467   | 44.4753        | 26.4341                     | 0.4512          |
| Taurocholic acid | 175.9461 | 1.0765    | 34.8788        | 20.0363                     | 0.1243          |
| Taurohyodeoxycholic acid | 12.4800 | 0.1096    | 5.4691         | 3.3819                      | 0.0171          |
| Taurothocholic acid | 0.0002 | 0.0011    | 0.0063         | 0.0040                      | 0.0003          |
| Tauroursodeoxycholic acid | 12.8510 | 0.1347    | 5.6816         | 3.5868                      | 0.0168          |
| Tauroxycholic acid | 3.9186 | 0.0321    | 1.0945         | 1.9210                      | 0.0099          |
| Taur-o-α-muricholic acid | 0.3432 | 0.0212    | 0.1269         | 0.1367                      | 0.0075          |
| Taur-o-β-muricholic acid | 0.0154 | ND        | 0.0926         | 0.0207                      | 0.0002          |
| Norcholic acid | 0.0464 | 0.0081    | 0.0284         | 0.0364                      | 0.0212          |
| Total:        | 403.4351 | 3.8244    | 260.4637       | 194.5035                    | 6.2923          |
ISOLATED CHOLESTASIS IS MORE LIKELY ASSOCIATED WITH NONSEVERE MUTATIONS IN MYO5B

A review of our patients with MYO5B deficiency and those reported by others identified 15 patients from 13 unrelated families with isolated cholestasis and 39 patients from 38 unrelated families with MVID. We compared MYO5B-associated isolated cholestasis and MVID based on nature of mutations by severity or by presumed effect on MYO5B-RAB11A interaction. We found that biallelic severe mutations were less frequent in the isolated cholestasis subgroup than in the MVID subgroup (0 of 13 vs. 11 of 38; \( P = 0.046 \); Fig. 5) as were biallelic mutations affecting the MYO5B-RAB11A interaction domain (5 of 13 vs. 28 of 38; \( P = 0.041 \)).

Discussion

BIALLELIC MUTATIONS IN MYO5B CAUSE ISOLATED LOW-GGT CHOLESTASIS

Although most cases of hereditary cholestasis in patients with low GGT levels can be explained by deficiencies in known genes (Supporting Table S1), many remain unexplained. In this study, approximately
one fifth (7 of 31) of the first two cohorts of undiagnosed low-GGT cholestasis patients carried biallelic mutations in MYO5B—mutations that either had been reported as pathogenic in other patients or are predicted in silico to be pathogenic (Table 1)—but not in genes known to be mutated in cholestasis (Supporting Table S5). Histologically, we observed coarse granular marking for MYO5B (Fig. 2) and disruption of canalicular distribution of BSEP (Fig. 3; the latter can be observed in some instances of primary BSEP deficiency owing to ABCB11 mutation). Among the 10 MYO5B-mutated patients identified in this study, 2 presented with persistent cholestasis, 3 with recurrent cholestasis, and 2 with transient cholestasis, a clinical spectrum also resembling those observed in ABCB11 mutation and in ATP8B1 mutation.

Similar plasma bile acid profiles were observed in patients with MYO5B mutations and in patients with ABCB11 mutations, consistent with involvement of BSEP malfunction in the cholestasis of MYO5B disease (Fig. 4). Mutated MYO5B in 5 children with low-GGT cholestasis and without MVID has been reported; however, the proportion of MYO5B mutation among children with genetically undiagnosed low-GGT cholestasis was not described in that report.

Our study suggests that, among Han Chinese patients, MYO5B defects account for a substantial proportion of hitherto undiagnosed hereditary low-GGT cholestasis.

The MYO5B/RAB11A apical recycling endosome pathway is important for canalicular biogenesis, formation of the canalicular membrane, and establishment of polarity in hepatocytes through transcytosis. BSEP expression is aberrant in typical MVID patients. The abnormal expression of BSEP and the change in bile acid profiles observed in our MYO5B-mutated patients suggest that MYO5B disease and ABCB11 disease share impaired bile acid secretion attributed to lack of functional BSEP in the canalicular membrane.

**GENOTYPE-PHENOTYPE CORRELATION IN MYO5B DISEASE**

Frequency of biallelic severe MYO5B mutations and of MYO5B mutations predicted to affect the MYO5B-RAB11A interaction domain differ between patients with isolated cholestasis and those with MVID. Isolated cholestasis appears to reflect relatively mild MYO5B functional deficiency, whereas severe mutations in MYO5B cause MVID. Cholestasis may accompany MVID; it was reported in 8 of 28 MVID patients in one study. However, most MVID patients are not noticeably cholestatic. This may be because the MYO5B-associated cholestatic phenotype in MVID shows the same kind of variability that we describe in our patients. For example, it is noteworthy that our patients P3, P8, and P9 have the MYO5B mutations c.1021C>T, p. Q341X and c.3046C>T, p.R1016X reported as associated with typical early-onset MVID, and that the severity of liver disease varies even between the brothers P1 and P2 (cf. clinical features in Results, above). Cholestatic phenotypes associated with MYO5B mutation thus appear to depend on modifier genes or possibly also on unknown environmental factors or epigenetic changes. In this context, the existence of a mouse model for MYO5B disease will provide a useful platform for testing mechanistic hypotheses. Such engineered mice may provide an experimental model to delineate the functional role of myosin Vb in the enterohepatic circulation of bile acids and may provide valuable clinical insights.

Among Han Chinese children, defects in MYO5B account for around 20% of instances of idiopathic low-GGT intrahepatic cholestasis. Cholestasis associated with MYO5B mutation need not be paired with persistent watery diarrhea and may be transient, recurrent, or progressive. A lack of severe biallelic MYO5B mutations in MYO5B associated cholestasis without diarrhea suggests that cholestasis is a manifestation of relatively mild MYO5B functional deficiency.

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**Appendix: URL Resources**

- GeneCards. http://www.genecards.org/
- Orphanet. http://www.orpha.net/consor/cgi-bin/index.php
- JuniorDoc. http://www.drwang.top/
- ClinVar. http://www.clinvar.com
- OMIM. http://www.omim.org
- PubMed. http://www.pubmed.org/
- HGMD. http://www.hgmd.cf.ac.uk/ac/index.phpdbSNP. http://www.ncbi.nlm.nih.gov/projects/SNP/index.html
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Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29020/suppinfo.