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

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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).[1-4] Of children with such cholestasis and low or normal serum gamma-glutamyltransferase (GGT) activity (GGT <100 IU/L; "low-GGT cholestasis"),[1] two thirds carry either *ATP8B1*[5,6] or *ABCB11* mutations[7-9]; the remaining instances of childhood cholestasis with conjugated

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine transaminase; ARC, arthrogryposis-renal dysfunction-cholestasis; AST, aspartate aminotransferase; BRIC, benign recurrent intrahepatic cholestasis; BSEP, bile salt export pump; CK, cytokeratin; DB, direct bilirubin; EDTA, ethylenediaminetetraacetic acid; FXR, nuclear farnesoid X receptor; gDNA, genomic DNA; GGT, gamma-glutamyltransferase; H&E, hematoxylin and eosin; IHC, immunohistochemical; LT, liver transplantation; MVID, microvillus inclusion disease; MYO5B, myosin VB; P, patients; PFIC, progressive familial intrahepatic cholestasis; RAB11A, RAS-related GTP-binding protein 11A; TB, total bilirubin; TBA, total bile acids; TJP2, tight junction protein 2; TNC, transient neonatal cholestasis; UDCA, ursodeoxycholic acid; UPLC–ESI/MRM-MS, ultrahigh performance liquid chromatography–electrospray ionization/multiple reaction monitoring mass spectrometry.

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hyperbilirubinemia and low GGT can sometimes be attributed to mutations in *TJP2*,[10] which encodes tight junction protein 2 (TJP2); in genes involved in bile acid synthesis, including *HSD3B7*,[11] *AKR1D1*,[12] *CYP7B1*,[13] *AMACR*,[14] and *CYP27A1*[15]; in *VPS33B* and *VIPAS39*, which cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome[16,17]; and in *NR1H4*, encoding the nuclear farnesoid X receptor (FXR)[18] (see Supporting Table S1). However, approximately one fifth of such patients remain without an identified genetic defect,[10] suggesting that mutations at additional loci are responsible for childhood low-GGT cholestasis. Myosin VB (MYO5B), associated with plasma membrane recycling and transcytosis,[19,20] 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.[21,22] The interaction of MYO5B with RAB11A also appears essential for targeting bile salt export pump (BSEP) to the canalicular membrane [23].

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.[24-27] 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[28-30] and is associated with altered targeting of BSEP to the canalicular membranes of hepatocytes.[31]

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[32,33]; and parental DNA available. All patients

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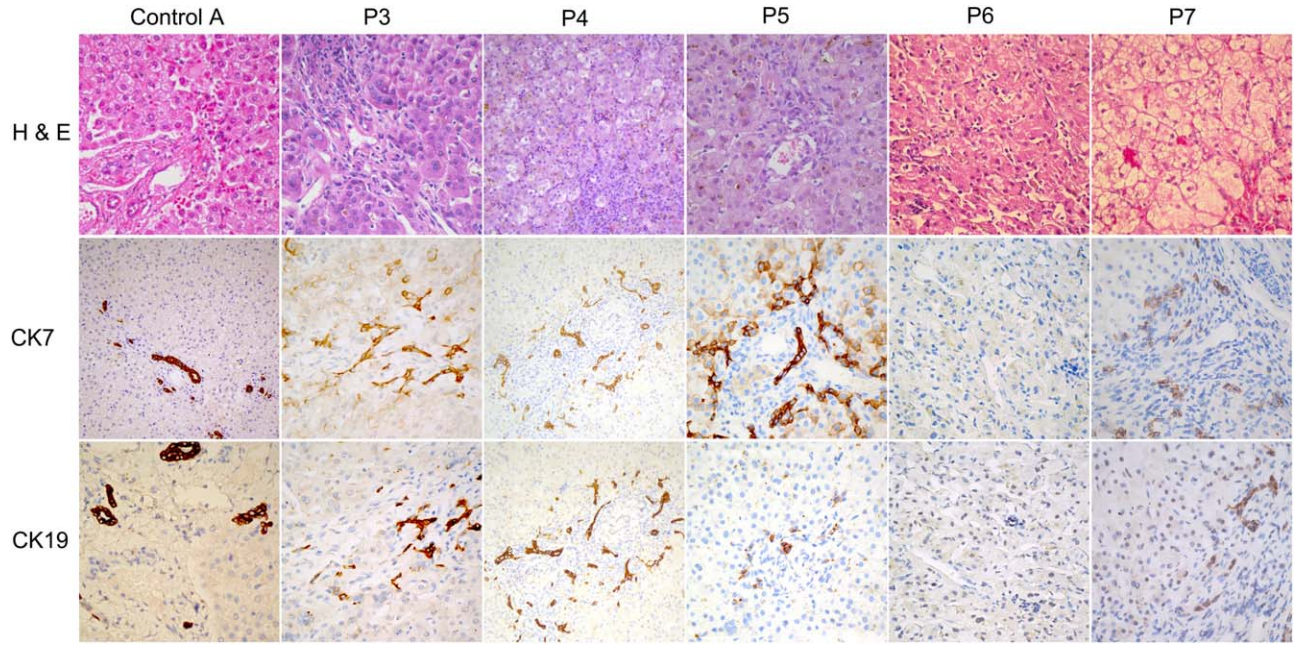

FIG. 1. Histological findings in *MYO5B* mutant patients (original magnification, all images, ×400). On H&E staining, hepatocellular and canalicular cholestasis, lobular disarray, mild inflammation, and portal-tract fibrosis were apparent in all specimens. Giant-cell formation was observed in all patients, 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.

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 [P] 1-5), 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

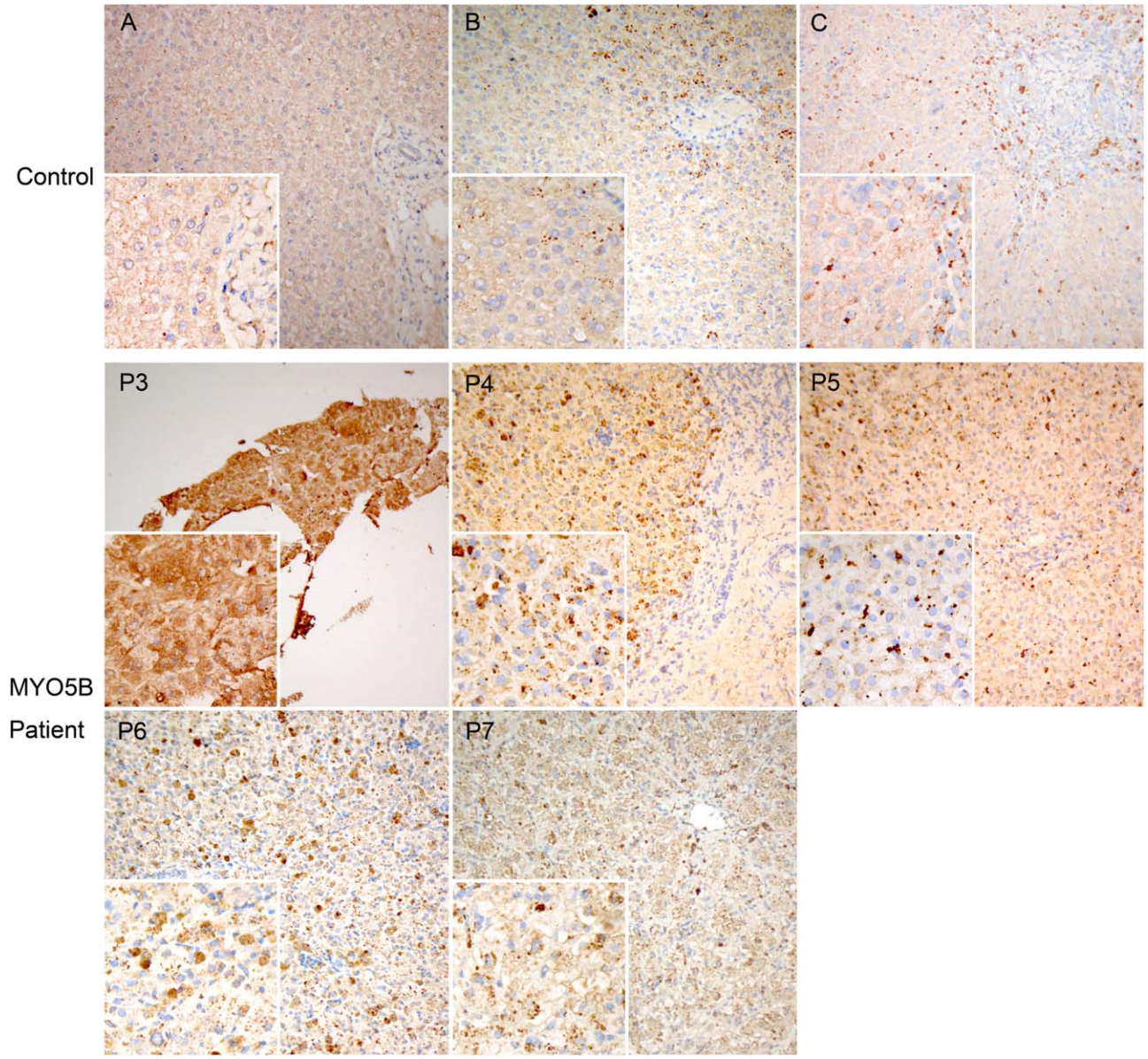

FIG. 2. MYO5B expression in *MYO5B* mutant patients (original magnification, all principal images, ×200; insets, ×400). (A) Choledochal cyst control without cholestasis; (B) incidentally resected normal liver control (adjoining excised tumor); (C) biliary atresia control with cholestasis. MYO5B Patients 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.

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

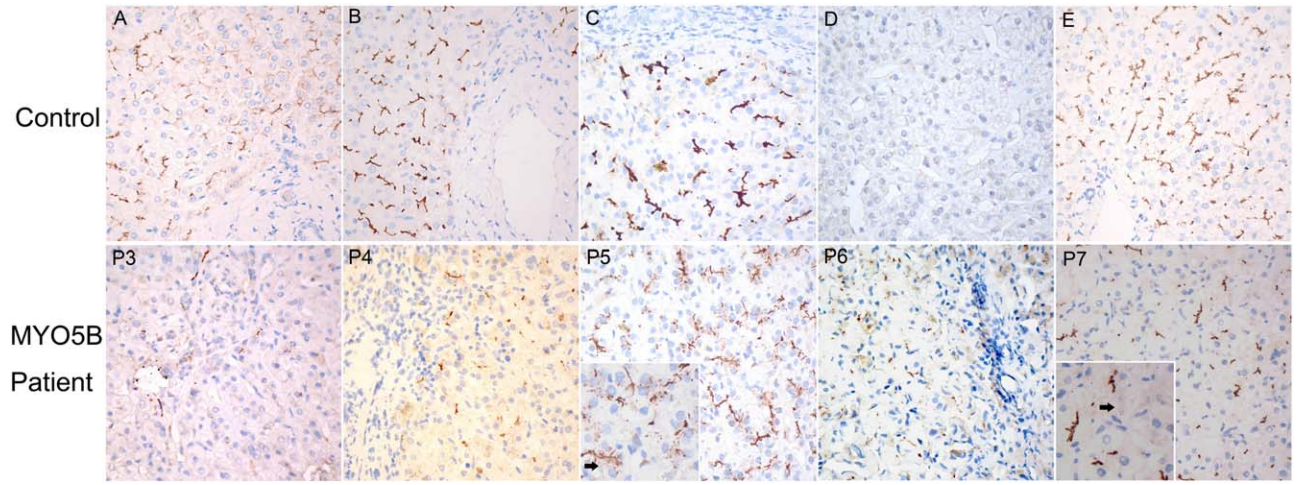

FIG. 3. BSEP staining in *MYO5B* mutant patients (original magnification, all principal images, ×400; insets, 900×). (A) Choledochal cyst control without cholestasis; (B) incidentally resected normal liver control (adjoining excised tumor); (C) biliary atresia control with cholestasis; (D) confirmed PFIC2 patient with biallelic *ABCB11* mutations (p.I498T / p.R415X); (E) discarded normal liver control (healthy liver donor). MYO5B patients P3-P5, P6, and P7: Compared to the control A and B, less expression of BSEP was observed in P3, P4, P6, and P7 (Fig. 3, P3-P4, P6, and P7), whereas expression was blurred at canaliculi and adjacent cytoplasm in P5 (Fig. 3, P5). Black arrows indicate abnormalities in P5 and P7 (insets).

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

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
	c.2414+5G>T	Splicing	IQ	Heterozygous	Mother	—	None	N/A	N/A
P3	c.1201C>T	p.R401C	Head	Heterozygous	Father	—	None	Deleterious	Probably damaging
	c.1021C>T	p.Q341X	Head	Heterozygous	Mother	CM108966[36]	Abolish interaction	N/A	N/A
P4	c.3237G>C	p.Q1079H	Coiled coil	Heterozygous	Father	—	None	Deleterious	Possibly damaging
	c.1604G>A	p.S535N	Head	Heterozygous	Mother	—	Abolish interaction	Tolerated	Possibly damaging
P5	c.796T>C	p.C266R	Head	Homozygous	Father and mother	—	Abolish interaction	Deleterious	Probably damaging
P6†	c.1748G>A	p.S583N	Head	Heterozygous	ND	—	Abolish interaction	Deleterious	Probably Damaging
	c.2801T>G	p.I934S	Coiled coil	Heterozygous	ND	—	Abolish interaction	Deleterious	Possibly damaging
P7†	c.2090_2090delG	p.R697Gfs*74	Head	Heterozygous	ND	—	Abolish interaction	N/A	N/A
	c.4852+11A>G	Splicing	Tail	Heterozygous	ND	—	N/A	N/A	N/A
P8 and P9	c.3046C>T	p.R1016X	Coiled coil	Heterozygous	Mother	CM085576[27]	Abolish interaction	N/A	N/A
P8 and P9	c.437C>T	p.S158F	Head	Heterozygous	Father	—	None	Deleterious	Possibly damaging
P10	c.2470C>T†	p.R824C	IQ	Homozygous	Father and mother	—	None	Deleterious	Probably damaging
Patients with monoallelic mutation									
P11	c.2470C>T†	p.R824C	IQ	Heterozygous	Mother	—	None	Deleterious	Probably damaging
P12	c.1136G>C†	p.R379P	Head	Heterozygous	Father	—	None	Deleterious	Possibly damaging

*MutationTaster assessed all mutations as disease-causing.
†While this article was under review, two additional *MYO5B* mutations causing isolated cholestasis were reported.[38] 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.

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).[40,41] 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 2. Clinical and Laboratory Attributes of MYO5B Disease Patients

Patients	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Sex	M	M	F	M	M	M	M	F	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	NA	50.1	47.1	68	NA	59.3	55.2
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 (<1%)	19 (70%)	9.5 (<3%)	13.1 (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

TABLE 2. *Continued*

Patients	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Alb	42	45	35.4	47	41.8	42.6	47.2	43	41	32.6
TBA	7.7	3.9	437.8	1.7	4.1	294.9	71.1	219.6	172.5	209.1
Outcome	Mild cholestasis with pruritus	Recovered	Listed elsewhere for LT and died aged 2.6y	Recovered with alleviated hepatomegaly	2 episodes of cholestasis with pruritus	Cholestasis	Cholestasis	In second episode of cholestasis with pruritus	Cholestasis with pruritus	In second episode of cholestasis with pruritus
Characterization	Persistent cholestasis	Transient cholestasis	Persistent cholestasis	Transient cholestasis	Recurrent cholestasis	Insufficient follow-up	Insufficient follow-up	Recurrent cholestasis	Insufficient follow-up	Recurrent cholestasis

*P4, P6, and P7 were too young (<3 months) to manifest itching-associated behavior at first assessment.
Abbreviations and (expected ranges), biochemical tests: TB, total bilirubin (5.0-17.1 umol/L); DB, direct bilirubin (0-6 umol/L); ALT, alanine transaminase (0-40 IU/L); AST, aspartate aminotransferase (0-40 IU/L); ALP, alkaline phosphatase (42-383 IU/L); GGT, gamma-glutamyltransferase (7-50 IU/L); TP, total protein (60-83 g/L); Alb, Albumin (35-55 g/L); TBA, total bile acids (0-10 umol/L).
Abbreviations: mo, month(s); d, days; NA, not available; y, years.

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

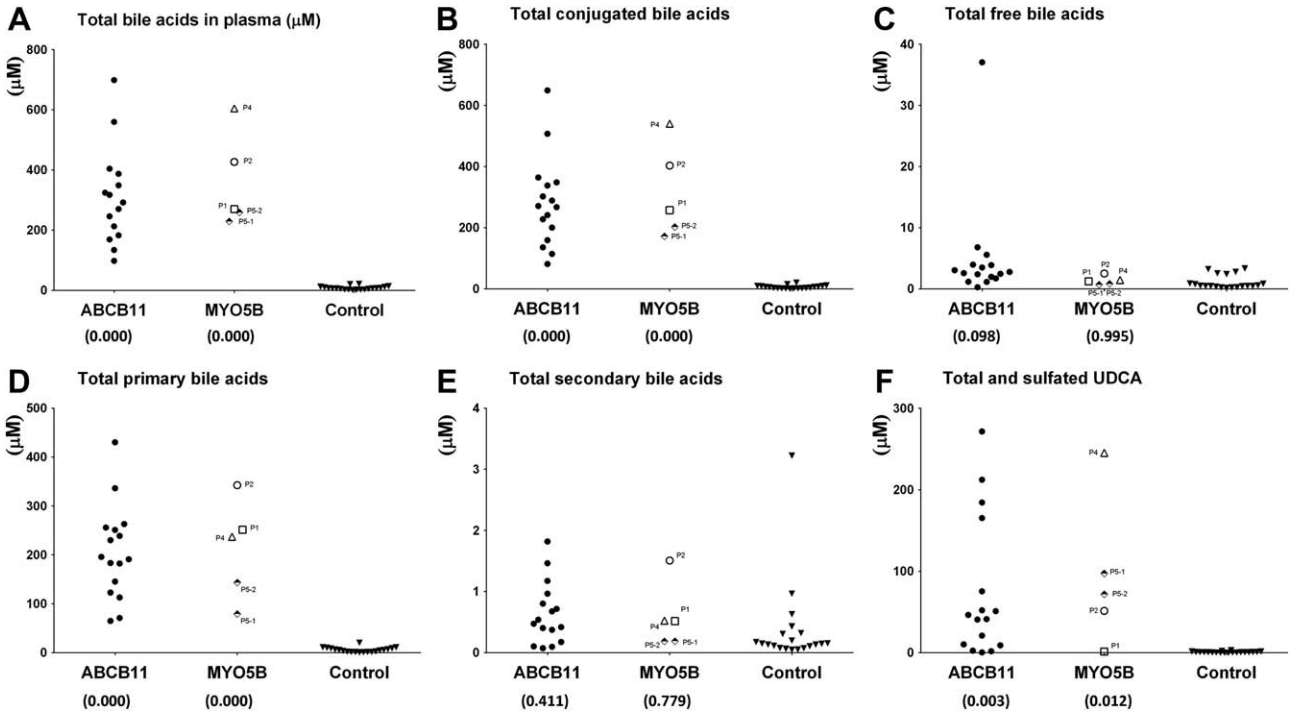

FIG. 4. Bile acids in plasma of *MYO5B* mutant patients. Bile acid (BA) profiles obtained by UPLC−ESI/MRM-MS (μM) of plasma from 4 patients carrying *MYO5B* defects, compared to those from 16 patients confirmed to harbor biallelic *ABCB11* mutations and 20 healthy controls. In the *MYO5B* group, P1, 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-DCA,tauro-CA, 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 DioxoLCA; (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.

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

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)

	Patient 2				
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
Nordeoxycholic acid	0.0004	0.0015	0.0010	0.0008	0.0007
λ-Muricholic acid	0.0030	0.0295	0.0018	0.0058	0.0135
ω-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
Taurochenodexycholic 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
Taurolithocholic acid	0.0042	0.0011	0.0063	0.0040	0.0003
Tauroursodeoxycholic acid	12.8510	0.1347	5.6816	3.5868	0.0168
Taurohyocholic acid	3.9186	0.0321	1.0945	1.9210	0.0099
Tauro-α-muricholic acid	0.3432	0.0212	0.1269	0.1367	0.0075
Tauro-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

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,[35] taurine-conjugated bile acid concentrations rose in P2 after resolution of cholestasis (Supporting Table S4). The significance of these differences is unknown.

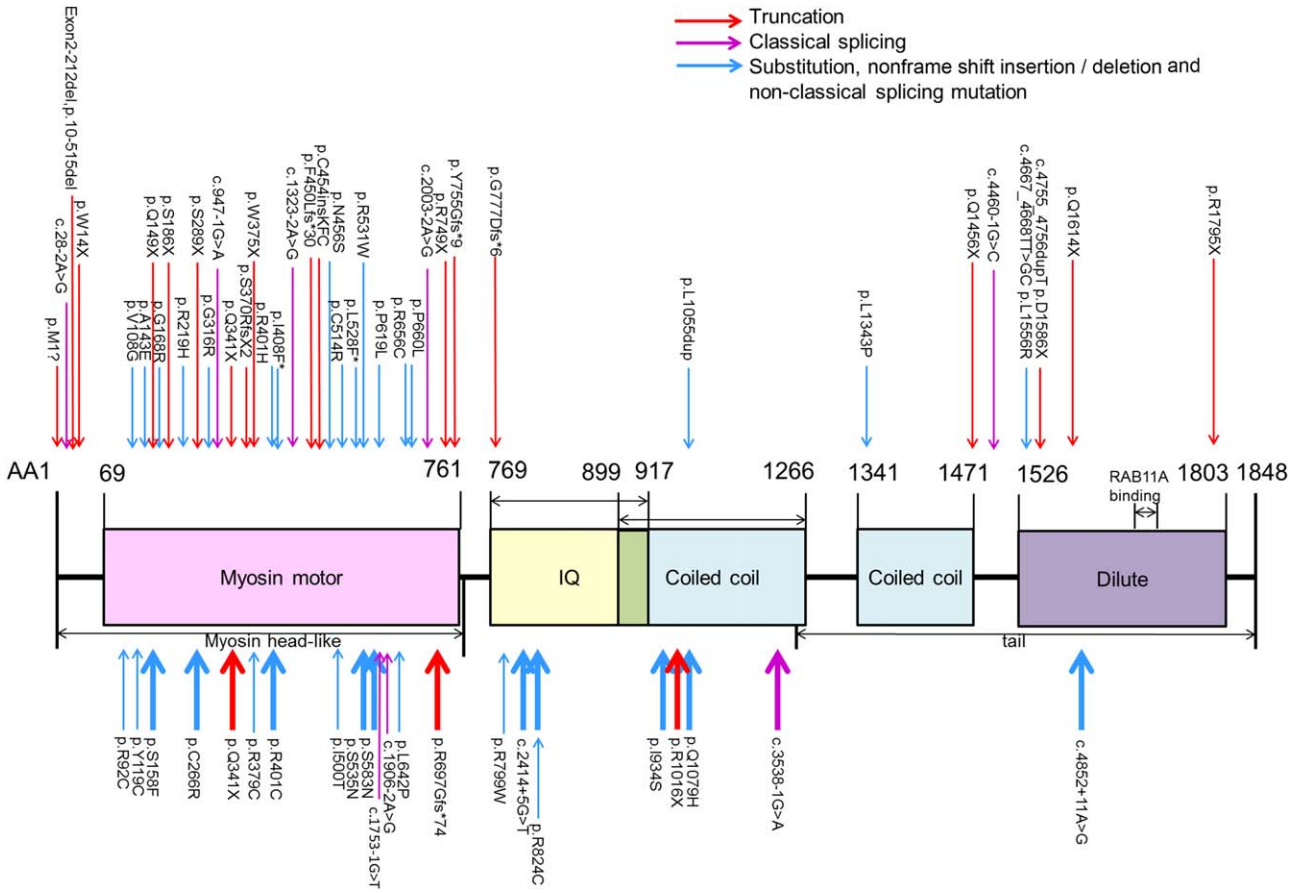

FIG. 5. Schematic presentations of MYO5B protein and reported homozygous or compound heterozygous mutations. Upper arrows indicate reported mutations in MVID patients.[24,25,27,29,36,37] Lower arrows show mutations in isolated-cholestasis patients reported in this article (thicker arrows) and elsewhere.[38] Protein data are deduced from UniProt (http://www.uniprot.org/uniprot/Q9ULV0). Severe mutations include truncations and classical splicing mutations. Abbreviation: IQ, Isoleucine-glutamine (IQ) calmodulin-binding consensus sequence. *Mutations p.I408F and p.L528F were reported in an MVID patient with "atypical" cholestasis.[29]

ISOLATED CHOLESTASIS IS MORE LIKELY ASSOCIATED WITH NONSEVERE MUTATIONS IN *MYO5B*

A review of our patients with MYO5B deficiency and those reported by others[24,25,27,29,36-38] 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.[10] 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).[33] 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.[6-9]

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[38] has been reported; however, the proportion of *MYO5B* mutation among children with genetically undiagnosed low-GGT cholestasis was not described in that report.[38] Our study suggests that, among Han Chinese patients, *MYO5B* defects account for a substantial proportion (~20%) 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.[23] BSEP expression is aberrant in typical MVID patients.[31] 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[31] 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[27] and c.3046C>T, p.R1016X reported as associated with typical early-onset MVID,[36] 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[26] 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.phpdb SNP. http://www.ncbi.nlm.nih.gov/projects/SNP/ index.html

Exome Variant Server (ESP6500). http://gvs-1.gs.washington.edu/EVS/
1000 Genomes http://www.1000genomes.org/
ExAC Browser. http://exac.broadinstitute.org/
These tools were applied in filtering common variants.
Polyphen2. http://genetics.bwh.harvard.edu/pph2/
SIFT. http://sift.jcvi.org
MutationTaster. www.mutationtaster.org

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Supporting Information

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