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Cholestasis Due to USP53 Deficiency

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See “Progressive Familial Intrahepatic Cholestasis: Is It Time to Transition to Genetic Cholestasis?” by Squires and Monga on page 641.

ABSTRACT

Objectives: Although a number of genetic forms of cholestasis have been identified, the genetic etiology of disease remains unidentified in a subset of cholestasis patients.

Methods: Whole exome sequencing (WES) was performed in DNA from patients diagnosed with cholestasis, at different points on the continuum from progressive familial intrahepatic cholestasis to benign recurrent intrahepatic cholestasis, in whom no disease mutations in known cholestasis genes had been identified. Candidate genes were then assessed in a larger patient sample, by targeted next-generation sequencing (NGS). Disease features at presentation and follow-up were collected from available medical records.

Results: By WES, we identified 3 patients with homozygous mutations in *USP53*. Screening of *USP53* in a larger set of patients identified 4 additional patients with homozygous mutations in *USP53*. Six of the 7 patients had deletion mutations, and 1 had a missense mutation; 3 of the patients were siblings, all bearing a deletion that also disrupted neighboring *MYO22*. Age of onset ranged from early infancy to adolescence. Cholestasis tended to be biochemically mild and intermittent, and responsive to medication. Liver fibrosis was, however, present in all 4 patients who were biopsied, and splenomegaly was apparent in 5 of 7 at last ultrasound.

Conclusions: Two groups recently identified patients with liver disease and mutation in *USP53*. We have now identified biallelic mutation in *USP53* in 7 further patients with cholestasis, from 5 families. Most individuals had evidence of chronic liver disease, and long-term follow-up is recommended.

Key Words: genetics, hepatology, intrahepatic cholestasis, liver, *MYO22*

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What Is Known

- Several distinct mechanisms are known to underlie genetic cholestasis.
- *USP53* is related to a family of ubiquitin-specific proteases, but may not function as one.
- *Usp53* mutation underlies a mouse deafness model.
- Mutations in *USP53* have recently been reported in early onset cholestasis.

What Is New

- Seven cholestasis patients with mutations in *USP53*.
- *USP53* deficiency may cause progressive liver disease.
- Age of onset is highly variable.
- Rifampicin may be useful as a treatment.

The manifestations of impaired bile formation are termed cholestasis; cholestasis can result from abnormalities in several different biological processes. The most prevalent defects are those affecting transporters involved in bile formation (1,2). Other cholestatic diseases are the result of abnormal development of the biliary tree (3,4). A third group of disorders are caused by ciliary dysfunction, reflecting the importance of cilia in regulation of bile flow (5–8). Patients with inborn errors of bile acid synthesis have been described (9). The normal organization and function of apical membranes is critical for bile formation and highly dependent on the correct trafficking of membrane constituents. Several cholestatic phenotypes are known to be the consequence of membrane abnormalities (10–14). Lastly, the structure of all epithelia is dependent on both cell/cell interactions and apical/basal polarization. Both functions are dependent on normal tight junction

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structures and tight junction-related forms of cholestasis have been described (15–17).

Most genetic causes of cholestasis ('cholestasis genes') have been identified through the investigation of children with early-onset disease. Many cholestasis genes so far identified, however, have now also been associated with later-onset forms of disease (18–21). In many cases, a degree of phenotype–genotype correlation has been found, with milder mutations leading to less severe phenotypes (22–25).

We performed whole exome sequencing (WES), and identified homozygous loss-of-function variants in Ubiquitin-specific protease 53 (*USP53*) in 3 cholestasis patients. Targeted screening of *USP53* in 1815 patients with early-onset or later-onset cholestasis then identified 4 additional patients harboring biallelic *USP53* variants likely to cause disease. Two groups have recently identified mutations in this gene, in patients with cholestasis (26,27).

PARTICIPANTS AND METHODS

Ethical Considerations

The study was approved by the King's College Hospital (KCH) Paediatric Liver Biobank, Childhood Liver Disease Research Network (ChiLDReN) site institutional review boards including UCSF, and the Ethics Committee of the Children's Memorial Health Institute (CMHI). The study was conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from adult participants and parents of each child.

Participants

WES was undertaken using DNA from 72 patients with genetically unexplained cholestasis, on the continuum from progressive familial intrahepatic cholestasis (PFIC) to benign recurrent intrahepatic cholestasis (BRIC). Forty-one of these patients were from the Longitudinal Study of Genetic Causes of Intrahepatic Cholestasis (LOGIC—NCT00571272) protocol. This study is organized under ChiLDReN, an NIDDK/National Institutes of Health-funded network of 16 pediatric academic medical centers across North America. LOGIC is a longitudinal observational study of the natural history of intrahepatic cholestasis. Details of enrollment criteria have been published, but included cholestasis, and allowed study entry between birth and 25 years of age (28). DNA from 14 children from KCH, and 17 from CMHI, also underwent WES; all were under 18 years of age, and had intrahepatic cholestasis, with no other diagnosis at enrollment. Of the 72 patients, 7 were previously reported to have disease-causing mutations in Tight Junction Protein 2 (*TJP2*) (17). For the targeted sequencing of *USP53*, 1249 children and 566 adults being investigated for cholestasis at KCH were examined. Clinical data of patients bearing *USP53* mutation was extracted from medical records or the ChiLDReN database.

Genetic Analysis

Genomic DNA was isolated from peripheral blood, or EBV-transfected cell lines, of patients and available parents. WES was performed, along with annotation and analysis as described previously (17). Subsequent analysis of variants identified by WES and targeted sequencing incorporated the use of Sorting Intolerant from Tolerant (SIFT), PolyPhen-2, Combined Annotation Dependent Depletion (CADD), and the Genome Aggregation Database (gnomAD) resources (29–33).

Once the initial patients with *USP53* mutation had been identified by WES, *USP53* was added to the NGS targeted gene panel used in the diagnostic/research laboratory at KCH. *USP53* was not analyzed for clinical diagnostic purposes, as it remained unproven as a cause of cholestasis; however, the *USP53* sequence data were extracted and analyzed as part of this project. Large deletions, identified by NGS, were confirmed by quantitative PCR (qPCR) of genomic DNA.

Histologic and Immunohistochemical Studies of Liver

Archival material obtained for the purposes of clinical diagnosis and treatment, fixed in formalin and processed into paraffin wax by routine methods, from individuals with mutation in *USP53*, was sectioned at 4 μ m. Routine immunostaining was performed (BondMax, Leica Biosystems, Buffalo Grove, IL) with the antibodies used against *USP53* (Sigma, Gillingham, Dorset, UK), *TJP2* (LS Bioscience, Seattle, WA), gamma-glutamyl transpeptidase (GGT) (Abnova, Taipei City, Taiwan), and bile salt export pump (BSEP) (Sigma) proteins, using hematoxylin counterstaining.

Ultrastructure Study

Tissue specimens were primarily fixed in paraformaldehyde and glutaraldehyde, post-fixed in osmium tetroxide and embedded in resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Whole-exome Sequencing

Potentially disease-causing variants in *USP53* were identified by WES in 3 patients. A homozygous >105 kb deletion was identified in 2 siblings (patients 1 and 2). The deletion breakpoints have not been sequenced but this deletion included the first coding exon of *USP53*, and all 5 coding exons of *MYO2Z*. Quantitative PCR (qPCR) of genomic DNA at multiple sites across the region confirmed homozygosity for this deletion in patients 1 and 2, and demonstrated heterozygosity in both parents. A third sibling (patient 3), born subsequent to the above discoveries, was found to be homozygous for the same deletion by qPCR and NGS panel analysis of the target regions (Table 1).

By WES, patient 7 was found to have a homozygous single nucleotide deletion in *USP53*, (c.510delA), predicted to result in a frameshift and subsequent protein truncation, p.(Ser171ArgfsTer62).

Targeted Gene Panel Sequencing

In a large group of patients with cholestasis in whom targeted gene panel sequencing had been performed, *USP53* sequence data were retrospectively analyzed. To note, *USP53* was evaluated in all patients from whom data were available, including patients who received diagnoses of other genetic forms of cholestasis, through analysis of the gene panel results. Of 566 adults tested, none showed likely disease-causing variants. In 1249 children with data available, 3 further individuals were found to have potentially disease-causing variants in *USP53*. None of these 3 patients had likely disease-causing mutations in the known cholestasis genes included on the gene panel. Two unrelated individuals (patients 4 and 5) were both found to be homozygous for a deletion spanning an intron/exon boundary; c.145-11_167del. Patient 6 was homozygous for

TABLE 1. Genetic and clinical findings in 7 patients with USP53 deficiency

Patient	1	2	3	4	5	6	7
USP53 variant	Deletion of first coding exon	Deletion of first coding exon	Deletion of first coding exon	c.145-11_167del	c.145-11_167del	c.725C>T	c.510delA
Predicted effect	Deletion	Deletion	Deletion	Deletion	Deletion	p.(Pro242Leu)	p.(Ser171ArgfsTer62)
Zygoty	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous
Sex	Female	Male	Female	Female	Male	Male	Male
Ethnicity	Turkish	Turkish	Turkish	Middle Eastern	Middle Eastern	North African	South Asian
Onset of cholestasis	3 months, recurred at 11 months and 6 years	2 months	5 months, approximately 18 and 21 months	7 years, plus gallstones	Neonatal then at 2, 3, and 13 years	15, 16, and 18 years	Approximately 4 years
Earliest available biochemistry documenting cholestasis	3 months	2 months	7 months	N/A	13 years	15 years	21 years
Age	6.6/6.1	5.2/3.8	18.4/14.6	NK	3.9/1.9	3.4/-	6.3/2.8
TB/DB, mg/dL	-/163	169/222	61/103	NK	22/38	78/74	84/46
ALT/AST, U/L	41	62	46	**Normal**	35	25	34
GGT, U/L	219	ND	ND	NK	37	ND	15
TBA, μmol/L	Normal	Normal	Normal	NK	NK	ND	NK
Audiometry	Normal	Normal	Normal	NK	NK	ND	NK
Liver and spleen USS	Liver normal Spleen	Liver normal Spleen	Liver normal Spleen	Both normal @ 10 years	Liver normal Spleen	Liver normal Spleen	Liver normal, GB wall thickened, contains sludge Spleen normal @ 20 years
Age	12.5 cm @11.5 years (enlarged)	10.2 cm @8.5 years	9.8 cm @2 years (enlarged)	10 years	10.5 cm @14 years (enlarged for weight)	15.5 cm @16 years (enlarged)	
Medication	None	none	2 years Rifampicin, UDCA. Relapses off Rifampicin	10 years UDCA	13 years Rifampicin	18 years None	21 years Rifampicin, UDCA and cholestyramine
TB, mg/dL	0.3	0.1	0.1	0.6	1.2	6.4	As above
ALT, U/L	40	48	40	47	17	50	
TBA, μmol/L	7	6	ND	ND	35	ND	

ALT = alanine aminotransferase; AST = aspartate aminotransferase; DB = direct bilirubin; GB = gall bladder; GGT = gamma-glutamyl transpeptidase; ND = not done; NK = not known; TB = total bilirubin; TBA = total bile acids; UDCA = ursodeoxycholic acid; ULN = upper limit of normal; USS = ultrasound scan. All USP53 variant positions are annotated on transcript: NM_019050.2.

c.725C>T, predicted to result in a missense change, p.(Pro242Leu). This variant is not present in the gnomAD database. SIFT predicts it to be deleterious, PolyPhen-2 predicts it to be damaging, and the scaled CADD score of 31 indicates that this mutation is very likely to deleteriously impact protein function. Pro242 is conserved, and only 14 amino acids distal to Cys228, the amino acid altered in the *mambo* mouse deafness model (34). Both of these amino acid residues are present in the fingers subdomain of USPs, considered the primary site of ubiquitin interaction (35).

Phenotype of USP53-Deficient Patients

Patients 1, 2, 3, and 5 came to medical attention with cholestasis in early infancy. Patient 2 also had heart failure, attributed to extremely low vitamin D levels. Patients 4 and 6 had their first episodes of cholestasis at age 7 and 15 years. In patients 1–6 the initial cholestasis resolved, although the time to so doing varied between 2 and 6 months. Patients 1, 3, 5, and 6 have had several episodes of cholestasis. In patients 1, 2, 3 and 4, cholestasis had resolved biochemically at last follow-up, but patients 5 and 6 were last seen during a cholestatic episode. Patient 7 presented with jaundice, pruritus, diarrhea, and abnormal liver function tests at approximately 4 years of age, and was jaundiced with raised transaminases when seen at 21 years of age; information on disease course between ages 4 and 21 is not available (Table 1).

Biochemically, the earliest documented cholestasis was associated with hyperbilirubinemia between 3.4 and 18.4 mg/dL with minimal or moderate elevation of transaminases. GGT levels were normal. Serum bile acid levels were not evaluated systematically; a level of 219 μ mol/L was recorded in patient 1 in infancy, 37 μ mol/L in patient 5 at 13 years of age during an episode, and 15 μ mol/L in patient 7 at 21 years of age. Patient 4 was noted to have gallstones when she presented at 7 years. At last follow-up, 4 patients had enlarged spleen, by ultrasound measurement.

The only extrahepatic manifestation noted in these patients was the infant (patient 2) with heart failure. This child was 1 of 3 siblings whose homozygous disease-causing mutation also included complete loss of the gene *MYOZ2*. All 3 siblings had subsequent echocardiograms, which were normal. All available serum calcium levels at presentation and during follow-up were normal. Three of

the patients had hearing tests with normal results. None of the patients have reported hearing problems.

A variety of treatments were employed. At last follow-up, 4 of 7 patients were still receiving medication. One was on rifampicin alone, 1 was on ursodeoxycholic acid (UDCA) alone, 1 was on UDCA and rifampicin, and 1 was on rifampicin, UDCA and cholestyramine. The patient on UDCA and rifampicin had relapse of pruritus twice when medication was withdrawn, with prompt amelioration of pruritus on reintroduction of rifampicin.

Histopathologic, Immunohistologic, and Ultrastructural Findings

Liver biopsy material was available from 4 patients, who underwent liver biopsy ages 2 months and 19 days (patient 2), 4 months and 23 days (patient 1), 13 years 2 months (patient 5), and 16 years 6 months (patient 6). All biopsies demonstrated fibrosis, ranging from portoseptal fibrosis in patients 1, 2, and 6 (Figs. 1 and 2) to portoportal bridging fibrosis (patient 5, Fig. 2). Portal tracts in all patients demonstrated a mild ductular reaction. Cholangiopathic features were not identified. One patient (patient 2, Fig. 1) demonstrated neonatal giant cell hepatitis. In the remaining patients, the liver lobule showed minimal disorganization, with the exception of biliary rosette formation in patient 1. All demonstrated mild lobular activity. Canalicular and hepatocellular cholestasis was variably seen. Canalicular proteins GGT and BSEP were expressed in a panlobular distribution in all patients. TJP2 was expressed in all patients. USP53 immunostaining was attempted but a meaningful expression pattern was not achieved.

Material was available for electron microscopy from patients 1, 2, and 5. A number of features were found (Fig. 3). In all 3 cases, some canaliculi were distended with bile whilst others contained healthy looking microvilli. In patient 1, the retained bile was coarsely granular (panel A), but that seen in patients 2 and 5 was finely filamentous. Patient 1 showed marked peri-canalicular filamentous actin (panel A). Abnormality of some desmosomes was noted in patient 2. They were either strikingly abnormal in length, or apparently detached from the plasma membrane (panel B). No abnormalities of tight junction morphology were identified in any patient. It is not clear if the changes noted are primary consequences of USP53 deficiency, or are secondary to the disease.

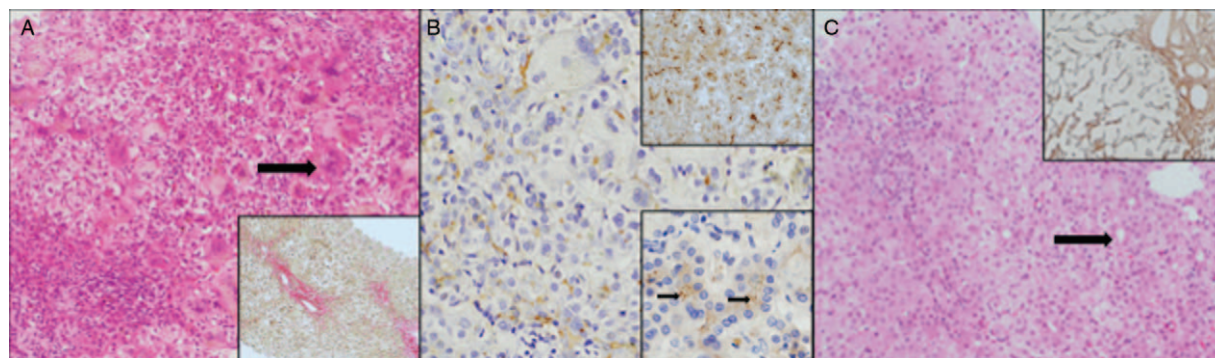


FIGURE 1. Patient 2 underwent liver biopsy ages 2 months and 19 days. Portoseptal fibrosis was present (A, inset, picro sirius red \times 100 magnification). The liver demonstrated hepatocyte giant cell change (A, main image, H&E \times 200 magnification, arrow) canalicular and hepatocellular cholestasis and mild lobular activity. Immunostaining demonstrated panlobular GGT expression (B, main image, \times 200 magnification), panlobular BSEP expression (B, upper inset \times 200 magnification) and canalicular TJP2 (B, lower inset \times 200 magnification, arrows) demonstrating accentuation at the tight junction. Patient 1 underwent biopsy ages 4 months and 23 days of age. The liver demonstrated portoseptal and perisinusoidal fibrosis (C, inset, reticulin \times 100 magnification). Biliary rosette formation was present, but the lobule was otherwise unremarkable (C, main image, H&E \times 100 magnification, arrow).

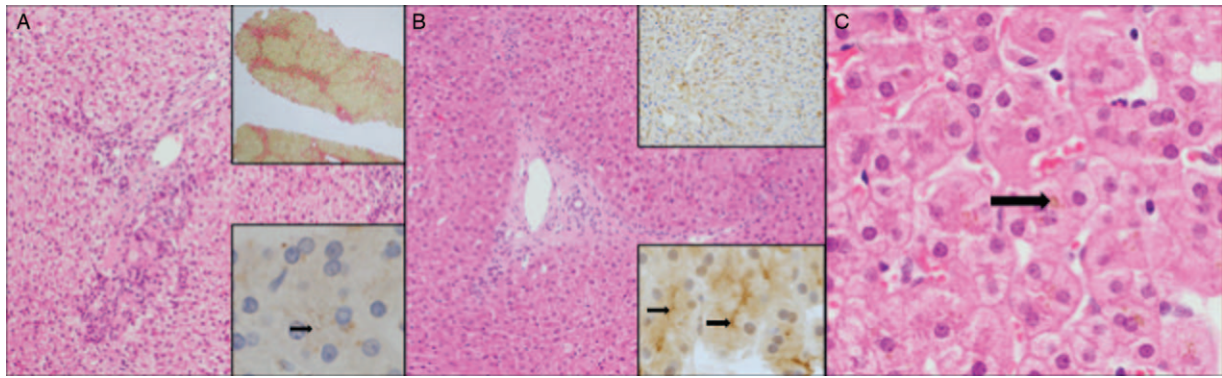


FIGURE 2. Patient 5 was biopsied ages 13 years and 2 months. The liver demonstrated portoportals bridging fibrosis (A upper inset, picro Sirius red $\times 40$ magnification). There was no significant disorganization of the lobular architecture (A main image, H&E $\times 100$ magnification). TJP2 expression was focal and of low intensity (A inset, $\times 400$ magnification). Patient 6 was biopsied ages 6 years and 6 months of age. The liver demonstrated portoseptal fibrosis (B, main image, H&E $\times 100$ magnification). There was panlobular expression of canalicular markers (B, upper inset shows GGT expression $\times 100$ magnification) and TJP2 was expressed at the canalicular membrane (B, lower inset, TJP2 immunostain $\times 400$ magnification). Very focal hepatocellular and canalicular cholestasis was seen (C, H&E $\times 200$ magnification with arrow pointing to hepatocellular cholestasis).

DISCUSSION

On the basis of sequence homology, *USP53* is predicted to encode a member of a family of proteins involved in de-ubiquitination, and therefore, important for regulation of protein degradation (36). *USP53*, however, lacks a histidine residue essential for de-ubiquitination activity, and is thought to have completely lost this function (36,37). In mice, *Usp53* has been shown to colocalize and interact with *Tjp2* (*ZO-2*) and, at least in the ear, contribute to tight junction structures (34). TJPs are cytoplasmic proteins that appear to link the intrinsic membrane tight junction proteins to the cytoskeleton (38,39). Variants in *TJP2* have been shown to underlie a range of liver phenotypes, from familial hypercholanemia to severe progressive cholestatic liver disease (15,17,40). In addition, loss of claudin 1, an intrinsic tight junction protein, has been shown to lead to a cholangiopathy (16).

The patients identified in this study presented with normal-GGT cholestasis, at a variety of ages from early infancy to adolescence. Four patients were demonstrated to have had recurrent episodes of cholestasis for which there have been no obvious precipitating factors, except

patient 3, who relapsed twice after stopping rifampicin administration. All patients were homozygous for their disease-causing variants. In 6 of 7 patients, the variant was predicted to cause a complete loss of function. One patient was, however, homozygous for a missense change; this individual presented at the oldest age, not manifesting any symptoms until 15 years of age.

Patients 1, 2, and 3 were homozygous for a deletion including exon 1 of *USP53* and the whole of *MYOZ2*. Heterozygous missense changes in *MYOZ2* have been associated with hypertrophic cardiomyopathy (HCM) (41). Mice heterozygous for the same changes also developed HCM (42). Mice with homozygous knockout of *Myoz2* did not show HCM, but manifested abnormalities of calcineurin signaling (43). One of the 3 siblings with the large deletion presented with heart failure. At the time this was attributed to hypovitaminosis D. All 3 siblings have subsequently had cardiac investigations with normal results. It is not clear whether the *MYOZ2* deletion and/or low vitamin D levels (even in presence of normal serum calcium levels) were the precipitant(s) of the heart failure seen in patient 2.

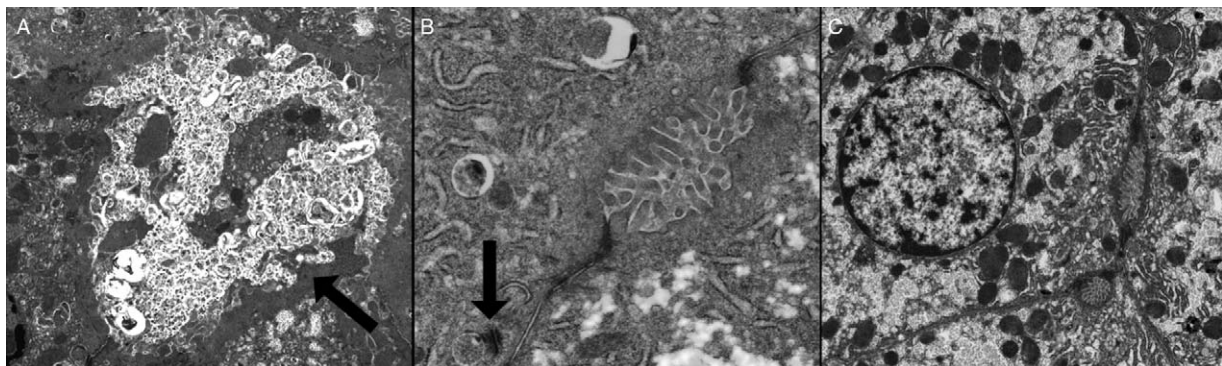


FIGURE 3. Transmission electron micrograph of biopsy taken from patient 1. Showing coarsely granular bile and pericanalicular filamentous actin, indicated by arrow (A, original magnification $\times 2600$). Biopsy from patient 2 showing relatively normal canalculus and desmosome apparently detached from plasma membrane, indicated by arrow (B, original magnification $\times 9200$). Biopsy from patient 5 showing unremarkable canaliculi and tight junctions (C, original magnification $\times 2600$).

A Middle Eastern family has recently been published in which 3 children with cholestasis, presenting at 1 year or less, were homozygous for a protein-truncating variant in *USP53* p.(Phe317-LeufsTer6) (26). Subsequently, 7 Chinese patients with onset of cholestasis <7 months of age, and biallelic mutations in *USP53*, were reported (27). In the former article, 2 of the children had bilateral hearing loss, all 3 had notable hypocalcemia, and 1 child received a liver transplant for intractable pruritus at age 6 years. In the latter article, 6 of 7 patients had normal bilirubin at last follow-up; 1 had severe early-onset deafness. Two Middle Eastern patients appeared responsive to rifampicin treatment, and 4 of 7 Chinese patients were on UDCA and/or cholestyramine at last follow-up. The Mambo mouse deafness model, harboring a homozygous missense change in *Usp53*, manifests cochlear hair cell loss (34). No other phenotype was described in these mice. Neither hearing loss nor hypocalcemia has been noted in any of our patients.

Our data, and the 2 previous reports, convincingly indicate that loss of *USP53* function is associated with normal-GGT cholestatic liver disease. In general, the clinical features appear relatively mild, and can be relapsing. The fibrosis seen in the liver biopsies, and the enlarged spleens in 4 patients, however, suggest that long-term follow-up is essential.

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