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ARTICLE

New paradigms of *USP53* disease: normal GGT cholestasis, BRIC, cholangiopathy, and responsiveness to rifampicin

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Abstract

Biallelic variants in the *USP53* gene have recently been reported to segregate with normal gamma glutamyltransferase (GGT) cholestasis. Using whole-exome sequencing (WES), we detected two *USP53* homozygous variants (c.951delT; p. Phe317fs and c.1744C>T; p. Arg582*) in five additional cases, including an unpublished cousin of a previously described family with intractable itching and normal GGT cholestasis. Three patients, a child and two adults, presented with recurrent episodes of normal GGT cholestasis, consistent with a diagnosis of benign recurrent intrahepatic cholestasis (BRIC). Cholangiopathic changes, possibly autoimmune in origin, were recognized in some patients. Additional phenotypic details in one patient included an enlarged left kidney, and speech/developmental delay. Notably, two patients exhibited a complete response to rifampicin, and one responded to ursodeoxycholic acid (UDCA). Two adult patients were suspected to have autoimmune liver disease and treated with steroids. This report describes new cases of USP53 disease presenting with normal GGT cholestasis or BRIC in three children and two adults. We also describe the novel finding of a dramatic response to rifampicin. The association of cholangiopathy with normal GGT cholestasis provides a diagnostic challenge and remains poorly understood.

Introduction

Hereditary cholestasis is a heterogeneous group of autosomal recessive liver disorders in which the production and release of bile acids from hepatocytes is impaired. A number of clinical conditions including progressive familial intrahepatic cholestasis (PFIC) can lead to pediatric cholestasis and progress to cirrhosis and end-stage liver disease before adulthood [1]. The diagnostic yield of genetic testing in cases of pediatric cholestasis currently ranges from 25 to 50%. However, this is expected to increase as more candidate genes are discovered and confirmed [1]. We recently reported the association of several novel loci with pediatric cholestatic liver disease [2, 3].

Benign recurrent intrahepatic cholestasis (BRIC) is characterized by recurrent episodes of cholestasis manifesting by jaundice, pruritus, anorexia, fatigue, and steatorrhea. Episodes can be spontaneous or triggered by infection or pregnancy and can last from weeks to months [4, 5]. Familial cases of BRIC affecting children and adults are caused by mutations in *ATP8B1* (BRIC1) [4–6], *ABCB11* (BRIC2) [7], *TJP2* [8], and *MYO5B* [9].

The *USP53* gene encodes a non-protease protein homolog of the ubiquitin-specific peptidase family [10]. The USP53 protein is expressed at high levels in the kidney and inner ear, with medium expression in liver and brain tissues [2, 11]. USP53, a tight junction (TJ) protein, co-localizes and interacts at the cellular TJs with other tight junction proteins 1 and 2 (TJP1 and TJP2). Mutations in the *TJP2* gene are known to cause familial hypercholanemia [8, 12], normal GGT cholestasis (PFIC4) [8, 13], BRIC [8], intrahepatic cholestasis of pregnancy (ICP) [14], cirrhosis in adults [14], hepatocellular carcinoma [14–16], and deafness [17, 18]. A missense variant

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in *USP53* (c.682T>A; p.Cys288Ser) that does not alter the co-localization with TJP2 has been reported to cause progressive hearing loss in mice [11]. *USP53* is amongst the recently described novel loci associated with normal GGT cholestasis that additionally displayed hearing loss [2, 19].

In this study, we describe two homozygous variants in *USP53*, one of which is novel, in five new patients; two presented with normal GGT cholestasis and four (including one previously reported by our group [2]) with BRIC. In addition, two patients (including one previously reported by our group) showed evidence of cholangiopathy characterized by a normal GGT level occurring in infancy [2] or adulthood. Furthermore, patients with USP53 disease (both normal GGT cholestasis and BRIC phenotypes) responded to rifampicin. We also report novel findings, including speech and developmental delay, and provide further description of clinical phenotypes caused by primary loss of USP53 and hypothesize a disease mechanism that relates the primary loss of USP53 to normal GGT cholestasis, BRIC, and deafness caused by primary loss of ATP8B1 (PFIC1) or TJP2 (PFIC4).

Materials and methods

Human subjects

Patients with negative exome sequencing of known cholestasis-related genes were further investigated for the presence of deleterious variants in candidate genes. Informed consent was obtained from all the study participants as per an approved institutional review board (IRB) protocol (TU MLT-2019-07 and KFSHRC RAC #2121053).

Molecular testing and variants curation

Exome sequencing (ES) and Sanger confirmation were performed as previously described [2]. Briefly, DNA libraries were constructed using Agilent SureSelect Target Enrichment Kit and sequenced on an Illumina Hiseq2000 platform. Variant calling was performed using the Genome Analysis Toolkit (GATK). Sequence variants were classified and reported following the HGVS nomenclature and ACMG guidelines [20].

Results

Family 1

Patient IV:4 is a 4-month old male who was born at term to consanguineous parents. He presented in a comatose state and found to have cerebral hemorrhage and vitamin K responsive coagulopathy (INRat 10.0). He also had normal GGT cholestasis (Table 1) associated with itching. His neurological exam was unremarkable. He has a strong family history of three affected relatives reported previously by our group (Fig. 1a) (File S1) [2].

Family 2

Patient III:1 is a 7-year old male who was born at term to consanguineous parents (Fig. 1b). He presented at the age of 18 months with recurrent episodes of jaundice, severe itching, and normal GGT cholestasis (Table 1). His episodes were precipitated by mild infection and lasted 2–4 weeks after which they resolved in response to treatment with UDCA. On examination, he had mild hepatosplenomegaly. There was evidence of an enlarged left echogenic kidney on ultrasound. However, renal function was normal. A liver biopsy showed mild lobular and portal inflammation and stage 2 fibrosis with septae formation. He was delayed in acquiring gross motor skills. His mother reported that he had delayed speech and language development with about 30% intelligible speech for unfamiliar listeners.

In both patients, WES revealed the same homozygous frameshift variant in *USP53* (c.951delT; p.Phe317fs), detected previously in the three family members of the first family (Fig. 1a) [2]. Patient III:1 (Fig. 1b) was genetically diagnosed at the age of 5 years. This frameshift variant, predicted to undergo nonsense-mediated decay, was notably absent in gnomAD and local exome databases, and fully segregated with the disease in both families.

Family 3

Patient V:1 (Fig. 1c) is a 6-month old female infant who presented with jaundice, itching, and normal GGT cholestasis (Table 1). Parents are first cousins. WES revealed a homozygous pathogenic nonsense *USP53* variant c.1744C>T; p. Arg582*. Her jaundice and itching disappeared and liver enzymes normalized after starting rifampicin and UDCA.

Her 35-year old father (Fig. 1c, IV:3) was well until 18 years of age, after which he developed recurrent episodes of jaundice, itching and elevated liver enzymes. His parents are consanguineous. At age 22 years, he was diagnosed with biliary obstruction because of jaundice and right upper quadrant (RUQ) pain. Investigations showed total/direct bilirubin (TB/DB) 87/65 µmol/L, alkaline phosphatise (ALP) 438 U/L, alanine aminotransferase (ALT) 54 U/L, aspartate aminotransferase (AST) 60, and GGT 41 U/L. An abdominal ultrasound showed gallstone with normal common bile duct calibre. Endoscopic retrograde cholangiopancreatography (ERCP) and sphincterectomy were

Table 1 Summary of the clinical and molecular characteristics of the study participants.

Family	1	1[a]	1[a]	1[a]	2	3	3	3
Patient ID	IV:4	IV:5	IV:8	IV:1	III:1	V:1	IV:3	IV:2
Sex	M	F	F	M	M	F	M	F
Age at presentation	4 m	15 m	5 m	1 year	18 m	6 m	18 years	16 years
Reported Phenotype	Coagulopathy, normal GGT cholestasis	Cholangiopathy, normal GGT cholestasis	Tetany, BRIC	Normal GGT cholestasis	BRIC	Pruritus, normal GGT cholestasis	BRIC, cholangiopathy	BRIC
Additional phenotype	None	Deafness	Deafness	None	Speech and developmental delay	None	None	Hypothyroidism
Affected gene	*USP53*	*USP53*	*USP53*	*USP53*	*USP53*	*USP53*	*USP53*	*USP53*
Variant	c.951delT; p.Phe317fs	c.951delT; p.Phe317fs	c.951delT; p.Phe317fs	c.951delT; p.Phe317fs	c.951delT; p.Phe317fs	c.1744C>T; p. Arg582*	c.1744C>T; p. Arg582*	c.1744C>T; p. Arg582*
Total Serum Bile acids (TSBA) (μmol/l)	115	NA	79	163	155	353	NA	NA
Total Bilirubin (μmol/l)	172	159	26	402	155	146	876	179
Direct Bilirubin (μmol/l)	158	132	20	298	85	142	680	128
ALT (U/L)	30	97	25	136	51	36	45	63
AST (U/L)	37	82	29	352	61	69	45	80
GGT (U/L)	23	35	39	30	24	23	39	23
ALP (U/L)	553	6316	4432	2557	719	504	939	727
Clinical course (current age)	Alive with native liver (2 years)	Alive, post-liver transplant (24 years)	Alive with native liver (17 years)	Alive with native liver (6 years)	Alive with native liver (7 years)	Alive with native liver (1year)	Alive with native liver (35 years)	Alive with native liver (18 years)

m months.

[a]These are relatives of patient 1 (IV:4) who were reported previously by our group [2]. Reference data: TSBA0-8.6 μmol/l, total bilirubin5-17 μmol/l, ALT 10–40 U/L, AST 10–40 U/L,GGT 0–30 U/L,ALP 250–350 IU/L. The biochemical data are obtained at the age of presentation. The clinical course data are obtained at the given current age.

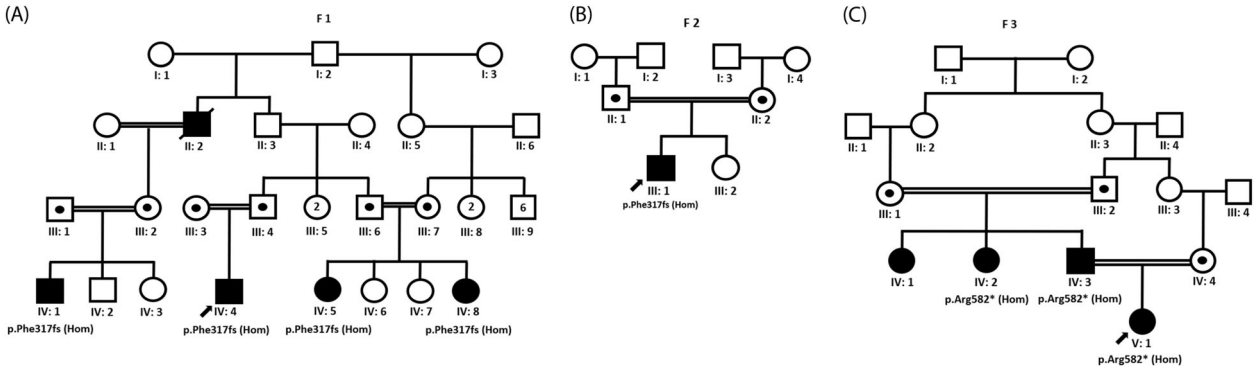

Fig. 1 Families with biallelic variants in *USP53* gene. Pedigrees for families 1 (A), 2 (B), and 3 (C) showing multiple affected individuals segregating the *USP53* variants in an autosomal recessive pattern.

performed, but found no evidence of gallbladder or biliary stones. He had temporary pancreatitis after ERCP. One month later, he presented with jaundice, intractable itching, pale stool, dark urine, poor appetite, loss of weight, and elevated liver enzymes (Table 1). He also had depression and suicidal ideation because of intractable itching. Liver biopsy revealed bland cholestasis. He responded gradually to treatment with UDCA and cholestyramine. His diagnosis was BRIC of unknown origin. He represented 4 years later with jaundice, itching, RUQ pain, fatigue and joint pain. Investigations showed TB/DB 532/492 µmol/l, ALP 588 U/L, ALT 55 U/L, and AST 62 U/L. Serological testing, including anti nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), liver-kidney-microsome (LKM), and anti-mitochondrial antibody (AMA) were all negative. Immunoglobulin and IgG4 levels were also normal. Repeat liver biopsy showed florid ductular proliferation, cholestasis, cholate stasis, feathery degeneration of hepatocytes, septal fibrosis, and mixed inflammatory infiltration (Fig. 2). He was diagnosed with autoimmune hepatitis overlapping with sclerosing cholangitis and subsequently started on steroids, UDCA, and cholestyramine. Azathioprine was added two months later. GGT was transiently elevated upon commencing the treatment. He represented one year later with a similar episode. High dose steroids were restarted and the azathioprine dose increased. He remained stable until 2020 when repeat magnetic resonance cholangiopancreatography (MRCP) showed no evidence of intrahepatic or extrahepatic biliary disease with early chronic paranchymal changes and stable splenomegaly (the same size as in 2008). Upon reaching a molecular diagnosis in his daughter, targeted mutation analysis confirmed homozygosity for the same variant thus confirming the pseudodominant inheritance of the disease in this highly inbred family.

The 18-year old youngest aunt (Fig. 1c, IV:2) is also affected by the same disease. She had three episodes of jaundice, intractable itching, and normal GGT cholestasis since age 16 years (Table 1). The first episode lasted 3 months. The second lasted 6 months associated with severe depression. ANA was 1/320 speckled. ASMA and AMA were negative. Ultrasound, MRI and MRCP of abdomen were unremarkable. Liver biopsy showed canalicular cholestasis with early ductular proliferation, but no fibrosis. She responded to tapering steroids. The last episode, that occurred 4 months ago, has improved with daily doses (10 mg) of prednisolone. She also takes thyroxin for hypothyroidism. Targeted mutation analysis confirmed homozygosity for the same variant that was found in this family.

The eldest aunt (IV:1) in the same family had cholecystectomy with history of recurrent jaundice and itching. However, there is no additional data available for this patient.

Discussion

PFIC is a group of cholestatic disorders (PFIC1-6) defined by genetic defects in bile acid transport or tight junction structure and associated with normal or high levels of GGT [1]. Mutations in several genes have been reported to cause PFIC [1]. We previously reported three children with a *USP53* homozygous variant [2]. We have added two more children with the same variant (c.951delT; p.Phe317fs), one from the same extended family (Fig. 1a) [2]. We also added three more patients, a female infant with her father and aunt, with a second homozygous variant (c.1744C>T; p.Arg582*). Clinical presentation in eight patients (including three in our previous report) included signs of fat-soluble vitamin deficiency (tetany and hypocalcemia due to vitamin D deficiency and intracranial bleeding due to vitamin K deficiency), normal GGT cholestasis resembling PFIC1 or 2, BRIC in children or adults (four patients, including one in our previous report), and a special form of cholangiopathy associated with normal GGT cholestasis [2]. The biochemical pattern of all eight patients is characterized by high bilirubin and ALP, mildly elevated ALT/AST, and

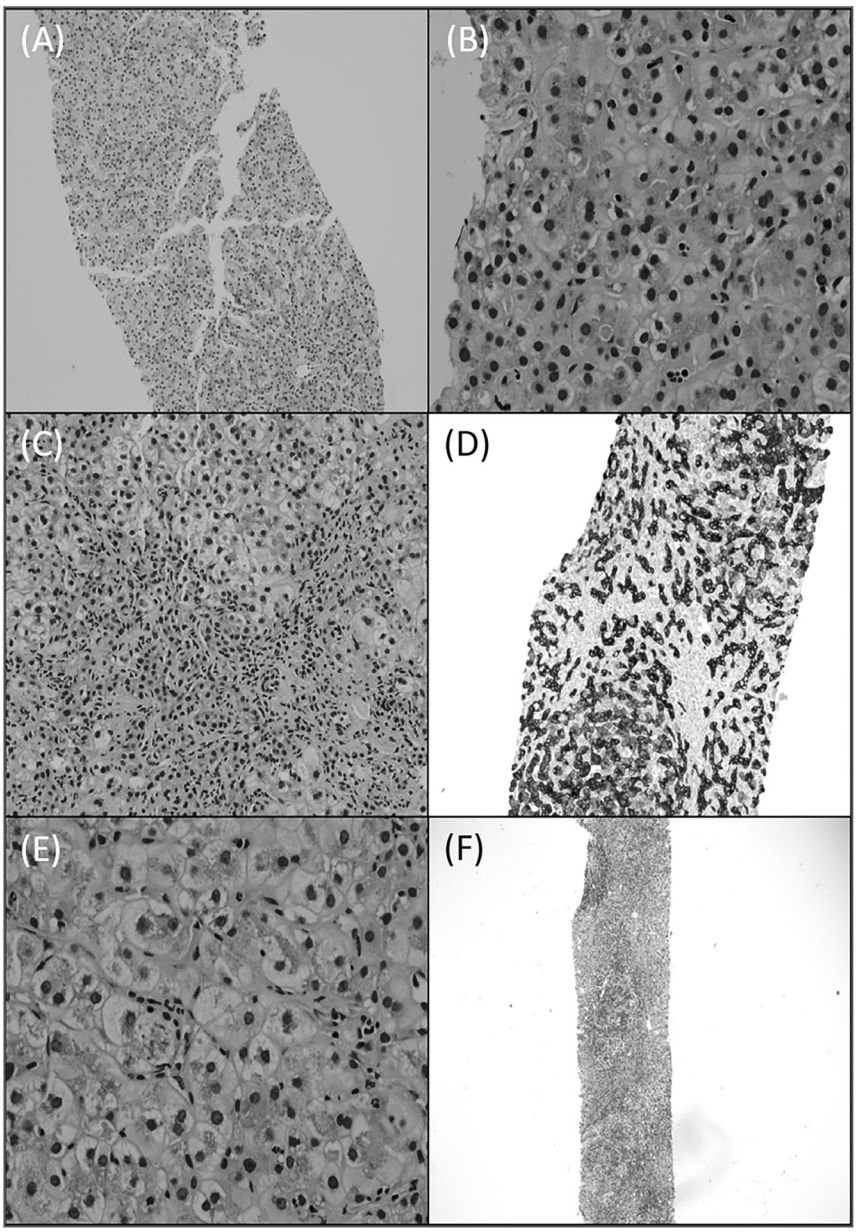

Fig. 2 Liver biopsy samples obtained in two BRIC occasions from patient IV:2 (family 3). a, **b** Liver biopsy during BRIC episode at age 22 years. **a** Preserved lobular architecture with no significant fibrosis (hematoxylin-eosin, magnification 40x). **b** The same sample show lobular bilirubinostasis with bile plugs in bile canaliculi without significant inflammation (hematoxylin-eosin, magnification 400x). **c–f** Liver biopsy during another BRIC episode at age 26 years. **c** Marked ductular reaction, with accompanying neutrophils. No loss of bile ducts (hematoxylin-eosin, magnification 100x). **d** Immunostaining with anti-cytokeratin 7 antibody highlights ductular reaction and show hepatocytes with aberrant expression, indicating biliary metaplasia. **e** Feathery degeneration of hepatocytes (cholate stasis)(hematoxylin-eosin, magnification 400x). **f** Focal portal-portal bridging fibrosis (Masson trichrome stain).

normal GGT (Table 1). Histopathologically, two showed cholangiopathy with florid duct proliferation and inflammation suggestive of biliary atresia in infancy [2] or sclerosing cholangitis in adulthood. Transient normal GGT cholestasis that responds to UDCA has been reported in seven other children [19]. Deafness has been reported in two children [2] and one neonate [19]. Unilateral large kidney and speech/developmental delay were observed in one child. Apart from Patient III:1 (Fig. 1b), no neurological abnormalities were reported in the other patients. We also report, in addition to two previous children [2], a remarkable response to rifampicin in two additional infants.

Interestingly, we recently reported a child with a homozygous variant in the *LSR* gene, which encodes a tricellular tight junction protein lipolysis-stimulated lipoprotein receptor (LSR), who presented with normal GGT cholestasis associated with speech and developmental delay [2]. A Japanese group reported another child with the same presentation, including speech and developmental delay [21]. It is possible that these developmental problems represent extrahepatic features of USP53 and LSR deficiency diseases, given that both tight junction proteins are highly expressed in the central nervous system [2, 11, 21]. Histopathological features of TJP2, USP53 (in this study), and LSR disease are comparable with each other [2, 13, 19, 21].

Biallelic variants in *TJP2* are known to cause normal GGT cholestasis (PFIC4) [8, 13], BRIC [8], ICP [14], and

deafness [17, 18]. Similarly, biallelic variants in *ATP8B1*, which lead to PFIC1, are known to cause normal GGT cholestasis [1], BRIC [4–6], ICP [1], and deafness [22, 23]. The overlap between ATP8B1 disease (PFIC1), TJP2 disease (PFIC4) and USP53 disease deserves special attention. Normal GGT cholestasis in PFIC1 is characterized by bland cholestasis [24] and mild increase of ALT/AST [25]. On the other hand, TJP2 disease may manifest with hypercholanemia [8, 12], bland cholestasis (resembling PFIC1), and inflammatory giant cell hepatitis (resembling PFIC2) (including personal observation of unpublished data on 20 cases with homozygous variants in *TJP2*). Hepatocellular carcinoma may occur in PFIC2 [26, 27] and PFIC2-like phenotype of TJP2 disease [14–16]. Indeed, double homozygous variants in *TJP2* and *ABCB11* have been shown to induce severe clinical phenotype or hepatocellular carcinoma [14]. Few of our USP53 phenotypes are characterized by PFIC1-like bland cholestasis. Some are reported to have inflammation, giant cell transformation, and fibrosis [19]. Others presented with BRIC characterized by bland cholestasis or obstructive-like cholangiopathy, both of which are associated with normal GGT. The initial liver biopsy at the age of 22 years in patient IV:3 (Fig. 1c) showed bland cholestasis. However, the second biopsy four years later revealed evidence of cholangiopathy and inflammatory hepatitis (Fig. 2). Similarly, the initial biopsy in patient IV:5 (Fig. 1a) at age of 15 months showed ductular proliferation and inflammation suggestive of biliary atresia [2]. Her initial presentation was at age 4 months. Normal GGT levels in spite of cholangiopathy may differentiate between USP53 disease and biliary atresia [2] or sclerosing cholangitis.

The reason for cholangiopathy that is associated with normal GGT is not clear. Possible mechanisms include; (1) initial bland cholestasis may extend with time to inflammatory cholangiopathy. Some BRIC cases are known to progress to persistent chronic cholestasis with normal GGT, forming two ends of the same spectrum [28]. However, cholangiopathy is not part of BRIC-PFIC spectrum. (2) Primary loss of TJP2 and USP53 were reported to cause secondary deficiency of another tight junction protein, the claudin1 (CLDN1) [13, 19]; primary deficiency of which is known to cause high GGT sclerosing cholangitis [29]. Secondary loss of CLDN1 may explain the mechanism of cholangiopathic changes observed in two patients. However, TJP2 and USP53 patients are not reported to have high GGT. Also, cholangiopathy, despite secondary loss of CLDN1, is not part of TJP2 disease. (3) The primary loss of USP53 may lead to recurrent attacks of biliary obstruction-like cholangiopathy. However, normal GGT and mildly elevated ALT/AST, despite severe jaundice and marked increase of ALP, characterize BRIC, but not obstructive cholangiopathy. In addition, large or small bile duct sludge or stones were not observed in our patients, although two had gallbladder stones. (4) The cholangiopathy induced by loss of USP53 is likely autoimmune in origin. However, the evidence for autoimmune cholangiopathy or hepatitis is not strong. The only supporting biochemical abnormality is related to high speckled ANA in the aunt. The response to steroids is not enough to define the disease as an autoimmune in nature. Therefore, the possible association with (or progression to) autoimmune phenomena warrants further confirmation. Interestingly, the biochemical pattern and pathological features of LSR disease are similar to that of USP53; both of which are characterized by cholangiopathy that associates with normal GGT cholestasis [2, 21]. Unlike our cases, GGT is usually high in most known cholangiopathies. Elevation of serum GGT in common cholangiopathies is thought to originate from secretion of this enzyme by cholangiocytes in response to injury from increased biliary bile acid concentration or toxic composition of bile. The exact mechanism that leads to the association or progression of BRIC to cholangiopathy, both of which are characterized by normal GGT, is obscure.

P4-ATPases are responsible for maintenance of membrane asymmetry by internalizing phosphatidyl serine into the inner leaflet of the plasma membrane, and are required for cell polarization [30, 31]. Similarly, TJs are known to play an important role in cell polarization [32, 33]. We hypothesize that primary loss of ATP8B1, TJP2, USP53, and LSR lead to overlapping phenotypes because of the same mechanism (Fig. 3). That is, permanent loss of membrane asymmetry caused by primary loss of ATP8B1 in PFIC1 may lead to secondary disruption of TJs, resulting thus in leakage of bile acids into serum. Bile acid leakage through disrupted TJs may explain the mechanism of hypercholanemia [34, 35] and lack of chronic inflammation in PFIC1 despite retention of bile acids due to secondary loss/inactivation of BSEP [36–38]. Secondary disruption of TJs in PFIC1 is probably dynamic in nature, which may increase or decrease according to the presence or absence of membrane stressors such as hydrophobic bile acids, oxidative stress, fever, infection, drugs, or hormones. Vice versa, primary loss of TJP2 or USP53 may lead to secondary inactivation (or loss) of ATP8B1, thus explaining the resemblance to PFIC1. In other instances, *TJP2* mutations may lead to TJP2 translocation into the nucleus [15] which may activate inflammatory genes while inactivating BSEP, resulting thus in inflammatory phenotype that resembles PFIC2. Indeed, double homozygous variants in *TJP2* and *ABCB11* were shown to induce a severe clinical phenotype or hepatocellular carcinoma [14]. Primary loss of USP53 may follow the path of TJP2; it may lead to bland cholestasis (i.e., PFIC1-like) or inflammatory giant cell hepatitis and cholestasis (i.e., PFIC2-like) (Fig. 3). A cholangiopathy associated with normal GGT may complicate

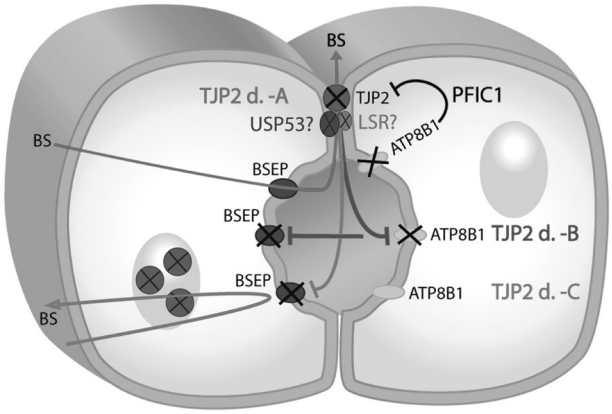

Fig. 3 Possible relation between ATP8B1, TJP2, USP53, and LSR. PFIC1; Permanent loss of canalicular membrane asymmetry caused by primary loss of ATP8B1, in the presence of membrane stressors, may lead to secondary disruption of TJs including TJP2. This may explain, in part, the physiology of PFIC1, including severe hypercholanemia and lack of hepatic inflammation. Primary loss/dysfunction of TJP2 may lead to three scenarios. TJP2 disease A; Bile acids secreted into the canalicular space by normal BSEP may leak back into serum through disrupted TJs, resulting in a hypercholanemia. TJP2 disease B; primary loss/dysfunction of TJP2 may lead to secondary loss/dysfunction of ATP8B1, and hence BSEP, resulting thus in a phenotype that resembles PFIC1 (non-inflammatory). TJP2 disease C; primary loss/dysfunction of TJP2 may lead to secondary loss/dysfunction of BSEP resulting thus in a phenotype that resembles PFIC2 (inflammatory). Primary loss of USP53 or LSR may induce, in certain aspects, a phenotype that resembles TJP2 disease.

USP53 disease. If numbering will continue to categorize diseases, then USP53 disease should be called PFIC type 7 (PFIC7). It appears that PFIC7 is the mildest among the four diseases. The transient nature of cholestasis [19], the response to UDCA and/or rifampicin, and the presentation with BRIC may all indicate the mild nature of the disease. This does not mean, however, that fat-soluble vitamin deficiencies were not severe or itching was not intractable; it mainly depends on how much bile acid is leaked back into the serum through disrupted TJs while not being able to reach to the gut. The main indication of liver transplantation in one of our previously reported patients (Fig. 1a, IV:5) was intractable itching that is not responsive to medical treatment (initially thought to be a biliary atresia). She may not have had the chance to try rifampicin 20 years ago. The severity of intractable itching during an episode of BRIC in two of our adult patients led to depression and/or generation of suicidal ideas.

The remarkable response to rifampicin in USP53 disease is interesting. Rifampicin activates pregnane X receptor (PXR)-regulated transcription of CYP3A4, which stimulates 6α-hydroxylation of bile salts that can be excreted through the basolateral membrane via MRP3 and/or MRP4 [39–41]. In addition, conjugation and excretion of bilirubin is enhanced through induction of UGT1A1 and MRP2 [39]. Rifampicin was shown to upregulate MDR1 expression through PXR signaling [42, 43]. Primary loss of Bsep in mice is compensated by upregulation of Mdr1 [44]. Rifampicin is known to reduce pruritus in most BRIC patients [5, 29, 41, 45–50], some of which were caused by mutations in *ATP8B1* [4–6, 29, 45, 47, 49]. We suspect that some of BRIC cases were caused by mutations in *USP53*. It will be interesting in the future to see the possible usefulness of rifampicin in preventing the recurrence of BRIC episodes, treating or preventing the progression into cholangiopathy, and possible modulation of neurological manifestations of USP53 and LSR-diseases. Some of our patients did not respond to UDCA; however all seven children reported by Zhang et al. were responsive to UDCA [19]. A combination of UDCA and rifampicin may yield a better response to various USP53 disease phenotypes. The possible progression into autoimmune reaction while responding to steroids in two adults is interesting. Two PFIC2 patients were previously reported to respond to steroids, possibly by upregulating ABC transporters [51]. Defining USP53 disease as a "familial steroid-responsive autoimmune cholangiopathy" requires further confirmation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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