ABSTRACT

Purpose: Progressive familial intrahepatic cholestasis (PFIC) is a rare genetic autosomal recessive disease caused by mutations in ATP8B1, ABCB11 or ABCB4. Mutational analysis of these genes is a reliable approach to identify the disorder.

Methods: We collected and analyzed relevant data related to clinical diagnosis, biological investigation, and molecular determination in nine children carrying these gene mutations, who were from unrelated families in South China.

Results: Of the nine patients (five males, four females) with PFIC, one case of PFIC1, four cases of PFIC2, and four cases of PFIC3 were diagnosed. Except in patient no. 8, jaundice and severe pruritus were the major clinical signs in all forms. γ-glutamyl transpeptidase was low in patients with PFIC1/PFIC2, and remained mildly elevated in patients with PFIC3. We identified 15 different mutations, including nine novel mutations (p.R470HfsX8, p.Q794X and p.I1170T of ABCB11 gene mutations, p.G319R, p.A1047P, p.G1074R, p.T830NfsX11, p.A1047PfsX8 and p.N1048TfsX of ABCB4 gene mutations) and six known mutations (p.G446R and p.F529del of ATP8B1 gene mutations, p.A588V, p.G1004D and p.R1057X of ABCB11 gene mutations, p.P479L of ABCB4 gene mutations). The results showed that compared with other regions, these three types of PFIC genes had different mutational spectrum in China.

Conclusion: The study expands the genotypic spectrum of PFIC. We identified nine novel mutations of PFIC and our findings could help in the diagnosis and treatment of this disease.

Keywords: Intrahepatic cholestasis; Phenotype; Gene; Mutation

INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of rare autosomal recessive liver disorders that present in infancy or early childhood as a cholestasis of hepatocellular origin and result in end stage liver disease and death or require liver transplantation (LT) [1]. Patients present with a wide-spectrum of clinical manifestations including pruritus, jaundice, growth failure, hepatomegaly, splenomegaly, and gradually...
progress to cirrhosis and end-stage liver disease. The exact incidence of PFIC is not precisely known but is estimated as 1 per 50,000 to 1 per 100,000 [2].

Based on the genetic defect in the hepatocellular transport-system gene involved in bile formation, PFIC is classified into three types: PFIC type 1 (PFIC1), PFIC type 2 (PFIC2), and PFIC type 3 (PFIC3) [3]. PFIC1, known as Byler disease, is caused by mutations in the ATP8B1 gene, resulting in the deficiency of familial intrahepatic cholestasis 1 (FIC1) protein. ATP8B1 gene is located on the human chromosome 18q21-22. ATP8B1 protein is a member of the type 4 subfamily of P type adenosine triphosphatase (ATPase) and is located on the canalicular membrane of hepatocytes. It acts as a flippase for aminophospholipid transport and leads to the movement of phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of the plasma membrane of hepatocyte. PFIC2, known as Byler syndrome, is caused by the mutations in the ABCB11 gene, resulting in the deficiency of bile salt export pump (BSEP). ABCB11 gene is located on human chromosome 2q24. BSEP is a transporter protein, expressed at the canalicular membrane of hepatocyte. It is the major exporter of bile acids from the hepatocyte to the canalicular against a concentration gradient. PFIC3 is caused by mutations in the ABCB4 gene, resulting in the deficiency of the multidrug resistant 3 (MDR3) protein. ABCB4 gene is located on human chromosome 7q21. MDR3 is a p-glycoprotein (pGp) and is a phospholipid translocator. It is expressed in the canalicular membrane of hepatocytes and acts as a floppase responsible for the biliary secretion of phospholipids, predominantly phosphatidylcholine.

PFIC accounts for 10–15% of the causes of cholestasis and 10–15% of LT indications in children [4,5]. PFIC1 and PFIC2 represent 2/3 of PFIC cases, whereas PFIC3 represents 1/3 of PFIC cases [6]. To date, PFIC has been reported in patients of Caucasian, Asian, and African origin [7-10]. In this study, we report the clinical features and gene analysis of nine Chinese patients with PFIC.

MATERIALS AND METHODS

Patients
Nine index patients (five males and four females) from nine unrelated families, clinically suspected of PFIC, were included in this study. They were referred to the Guangzhou Women and Children’s Medical Center (Guangzhou, China) between January 2011 and August 2017. The age of diagnosis ranged from 5 months to 5 years. The inclusion criteria were: symptomatic cholestasis with pruritus, jaundice, and increased levels of serum alanine aminotransferase (ALT) but low to normal levels of serum γ-glutamyl transpeptidase (γ-GT). Other causes of cholestasis including inborn errors in bile acid synthesis were excluded. Information related to demographics and family history, as well as regarding clinical manifestation, and biochemical analysis at onset (standard liver function tests and serum bile acid concentrations), were collected.

The degree of pruritus in the patients was evaluated as 0–4+, as described by Whittington and Whittington [11]: none=0; rubbing or mild scratching when not distracted=1+; active scratching without evident skin abrasions=2+; abrasions evident=3+; and cutaneous mutilation, hemorrhage and scarring evident=4+.

The study was approved by the Ethics Committee of the Guangzhou Women and Children’s Medical Center (2015-112). Informed consent was obtained from the parents of all patients.
Liver histology
Liver biopsy was performed in four patients after informed consent was obtained from the parents.

Genomic DNA sequence analysis
Genomic DNA was extracted from the peripheral blood of the patients and their parents using the Blood gDNA Magnetic Beads Purification Kit (Innovogen Inc., Brookline, MA, USA), according to the manufacturer’s instructions. Primers were designed using the Primer 5 software (Biosoft International, Palo Alto, CA, USA) and the sequences are listed in Supplementary Table 1. All the exons and intron/exon splice junctions of ATP8B1, ABCB11, and ABCB4 genes were amplified using PCR (Mastercyclers Pro TM Gradient Thermal Cycler; Eppendorf, Hamburg, Germany). PCR products were purified and sent to BGI (Beijing, China) for direct DNA sequence analysis (DNA Analyzer 3730; ABI, South San Francisco, CA, USA). Sequences were compared with the reference sequences using CHROMAS software (v.2.01; Technelysium Pty Ltd., Tewantin, Qld, Australia). The novel mutations in the study were determined by comparing the Human Gene Mutation Database (HGMD) and the National Center for Biotechnology Information database. Genetic variants were searched in the Single Nucleotide Polymorphism Database and the 1,000 Genomes Project. Novel mutations were verified through the direct sequencing of the PCR products obtained from samples of 100 unrelated healthy controls and compared to the Exome Aggregation Consortium database along with the NHLBI exome variant database.

Protein function prediction for novel mutations
To predict the effect of amino acid substitutions, we performed in silico analysis using the SIFT/PROVEAN (http://sift.jcvi.org) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) web software. SIFT score ranges from 0 to 1. The amino acid substitution is predicted as ‘damaging’ if the score is ≤0.05, and ‘tolerated’ if the score is >0.05 (J. Craig Venter Institute, La Jolla, CA, USA). The variant is predicted to be ‘deleterious’ if the PROVEAN score is ≤−2.5, and ‘neutral’ if the score is >−2.5 (J. Craig Venter Institute). The PolyPhen-2 prediction outcome could be either “probably damaging”, “possibly damaging”, or “benign”.

RESULTS

Clinical and biochemical manifestations of the patients
In the present study, of the nine patients (five males, four females) with PFIC, one case of PFIC1, four cases of PFIC2, and four cases of PFIC3 were diagnosed (Table 1). There was no history of consanguinity in any of the patients. The elder sister of patient no. 4, who had jaundice since 2 years of age, died at 6 years of age due to progressive cholestatic jaundice. The elder sister and brother of patient no. 8, who had liver dysfunction since 5 years of age, died at the age of 7 due to progressive liver cirrhosis. No similar family history was noted in other patients.

All children were born at term with a birth weight of above 2.5 kg. The onset age of the patients with PFIC1/PFIC2 ranged from a few days to 4 months after birth. However, the age at onset for PFIC3 spanned from a few days to 5 years after birth. Except patient no. 8, jaundice and severe pruritus were the major clinical signs in all patients which were subsequently persistent. Patient no. 8 presented with mild pruritus and was diagnosed with liver dysfunction through accidental laboratory examination. Patient no. 1 with PFIC1 presented with severe diarrhea. Impaired growth was observed in seven children. Weight for height z score was less than −2.
(wasting) in seven children, height for age z score was less than −2 (stunting) in four children, and both wasting and stunting were observed in four children. All patients had an enlarged liver (palpable liver below right costal margin 3–7 cm), while two patients had splenomegaly. All patients presented with normal neurological development.

All the patients except patient no. 8 had cholestatic jaundice upon first presentation, with markedly raised serum conjugated bilirubin levels. γ-GT was low or normal in the patients with PFIC1/PFIC2, and remained mildly elevated in the patients with PFIC3.

Four patients had undergone liver biopsies. Patient no. 1 with PFIC1 manifested mild cholestasis and lymphocytic infiltration in the portal areas. Patient no. 4 with PFIC2 showed mild cholestasis. Patient no. 6 and no. 8 with PFIC3 showed minimal or mild liver fibrosis.

Two patients had undergone LT. Patient no. 1 had undergone LT at the age of 2 years 8 months at the Beijing Friendship Hospital. However, she developed chronic graft failure and died of hepatic failure with severe infection before the second LT at the age of 6 years. Patient no. 4 received piggyback whole LT successfully at the age of 4 years 5 months in our hospital. He received an immunosuppressive regimen of tacrolimus (FK 506) after LT. The patient recovered well after LT and was followed up regularly. His liver function improved with normal ALT at 8 days after LT. His liver function was totally normal at 100 days after LT.

Except patient no. 1, the other two patients died of hepatic failure including patient no. 2 at 1 year 5 months and patient no. 9 at 10 months. Four patients did not appear for follow-ups including patient no. 3, 5, 6, and 7. Patient no. 4 received LT successfully. Patient no. 9 was still alive with liver cirrhosis, hepatosplenomegaly and delayed development.

**Genomic DNA sequence analysis**

Of the nine patients with significant variations, one had variations in the ATP8B1 gene, four in the ABCB11 gene, and four in the ABCB4 gene (Table 2). All these mutations were inherited from the parents (Fig. 1).

1. **ATP8B1 Mutations (PFIC1)**

There was only one patient with PFIC1 in this panel. Sequence analysis revealed two heterozygous mutations. One was a known missense mutation p.G446R while the other was a known deletion mutation p.F529del.
2. ABCB11 Mutations (PFIC2)

Among four patients with PFIC2, sequence analysis of ABCB11 revealed six different mutations including three missense mutations (p.A588V, p.G1004D, p.I1170T), two nonsense

Table 2. Clinical and genetic characteristics of nine patients with PFIC

| Patient no. | Type | Age at onset | Symptoms | Pathologic features | Outcome | Gene | Base change | AA change | Origin | Described |
|-------------|------|-------------|----------|--------------------|---------|------|-------------|-----------|--------|-----------|
| 1           | PFIC1| 4 d         | Jaundice, pruritus, hepatosplenomegaly, diarrhea | Cholestasis | Death after liver transplantation, 6 yr | ATP8B1 | c.1336 G>A | p.G446R | Father | Previously |
| 2           | PFIC2| 1 mo        | Jaundice, pruritus, hepatosplenomegaly | ND | Death, 1 yr 5 mo | ABCB11 | c.2380C>T | p.Q794X | Father | This study |
| 3           | PFIC2| 3 mo        | Jaundice, pruritus, hepatosplenomegaly | ND | Loss of follow-up | ABCB11 | c.1407delG | c.3011G>A | Father | This study |
| 4           | PFIC2| 4 mo        | Jaundice, pruritus, hepatosplenomegaly | Cholestasis | Liver transplantation | ABCB11 | Homozygous c.763C>T | p.A988V | Father | Previously |
| 5           | PFIC2| 2 mo        | Jaundice, pruritus, hepatosplenomegaly | ND | Loss of follow-up | ABCB11 | Homozygous c.3509T>C | p.I1770T | Father | This study |
| 6           | PFIC3| 11 mo       | Jaundice, pruritus, hepatosplenomegaly | Liver fibrosis | Loss of follow-up | ABCB4 | c.2489insA | c.3139_3141delGCAinsCC | p.T830NfsX11 | Mother | This study |
| 7           | PFIC3| 1 yr 7 mo   | Jaundice, pruritus, hepatosplenomegaly | ND | Loss of follow-up | ABCB4 | c.3955G>C | c.3220G>A | Father | This study |
| 8           | PFIC3| 5 yr        | Pruritus | Liver fibrosis, Pruritus, hepatosplenomegaly | Loss of follow-up | ABCB4 | Homozygous c.436C>T | p.P479L | Father | This study |
| 9           | PFIC3| 4 d         | Jaundice, pruritus, hepatosplenomegaly | ND | Death, 10 mo | ABCB4 | c.3143delA | c.3139G>C | Father | This study |

PFIC: progressive familial intrahepatic cholestasis, ND: not done.

Fig. 1. Pedigrees of the patients with PFIC in whom major variations in ATP8B1 (Patient no. 1), ABCB11 (Patient no. 2, 3, 4, 5) or ABCB4 (Patient no. 6, 7, 8, 9) genes were found. PFIC: progressive familial intrahepatic cholestasis.

2. ABCB11 Mutations (PFIC2)

Among four patients with PFIC2, sequence analysis of ABCB11 revealed six different mutations including three missense mutations (p.A588V, p.G1004D, p.I1170T), two nonsense
mutations (p.Q794X, p.R1057X) and 1 deletion mutation (p.R470HfsX8). Three novel mutations were found: p.R470HfsX8, p.Q794X, and p.I1170T.

3. ABCB4 Mutations (PFIC3)
Among four patients with PFIC3, sequence analysis of ABCB4 revealed seven different mutations including four missense mutations (G319R, p.P479L, p.A1047P, p.G1074R), one insertion mutation (p.T830NfsX11), 1 indel mutation (p.A1047PfsX8), and one deletion mutation (p.N1048TfsX). Six novel mutations were found: p.G319R, p.A1047P, p.G1074R, p.T830NfsX11, p.A1047PfsX8, and p.N1048TfsX.

Protein function prediction of novel mutations
The novel missense mutations p.I1170T in the ABCB11 gene, and p.G319R, p.A1047P, and p.G1074R in the ABCB4 gene were predicted as 'damaging' by the SIFT/PROVEAN and PolyPhen-2 web software.

DISCUSSION
Cholestasis is a major clinical sign in all the three forms of PFIC. The age of onset is usually within the first months of life and tends to appear earlier in PFIC1/2 than in PFIC3. In contrast to PFIC 1/2, clinical signs of cholestasis are observed within the first year of life in about one-third of cases in PFIC3 patients. PFIC3 may manifest mainly in childhood and even in young adulthood. The disease of PFIC3 usually progresses from chronic cholestasis with or without jaundice to portal hypertension and end stage liver disease as seen in patient no. 8. γ-GT was low or normal in patients with PFIC1/PFIC2, and remained mildly elevated in patients with PFIC3.

In this study, we studied a cohort of nine unrelated Chinese patients including one case of PFIC1, four cases of PFIC2, and four cases of PFIC3. Fifteen different mutations were identified, including nine novel mutations (p.R470HfsX8, p.Q794X, and p.I1170T in ABCB11 gene, p.G319R, p.A1047P, p.G1074R, p.T830NfsX11, p.A1047PfsX8, and p.N1048TfsX in ABCB4 gene) and six known mutations (p.G446R and p.F529del in ATP8B1 gene, p.A588V, p.G1004D, and p.R1057X in ABCB11 gene, and p.P479L in ABCB4 gene). The 15 mutations detected in the nine patients were mostly private mutations.

ATP8B1 encodes an amino-phospholipid flippase FIC1, which translocates phospholipids from the outer to the inner leaflet of the plasma membrane [12]. More than 100 different mutations have been detected in the ATP8B1 gene so far in patients with PFIC1 (HGMD at the Institute of Medical Genetics in Cardiff: HEXB Gene: http://www.hgmd.cf.ac.uk). Two known heterozygous mutations in the ATP8B1 gene were detected in patient no. 1 with PFIC1. The missense mutation p.G446R was first reported in compound heterozygous form with a nonsense mutation in a French patient [13]. The mutation p.G446R led to a substitution of the hydrophilic neutral glycine by hydrophilic alkaline arginine. A net charge alternation can adversely affect the structure and function of the FIC1 protein. Another deletion mutation p.F529del was previously reported in three patients: in homozygous form in a Japanese and an Indian patient, and in compound heterozygous form with a splice mutation in a patient of mixed Caucasian-African American ancestry [8,14,15]. The codons G446 and F529 were both involved in substrate binding and the related mutations may lead to an impairment of the enzyme activity. The study by Liu et al. [16] suggested that the linked mutation P209T and
IVS6+5G>T is a hot mutation in the Chinese population. However, both these mutations were not detected in our study.

ABCB11 encodes the BSEP, a liver-specific adenosine triphosphate (ATP)-binding cassette transporter [12]. So far, more than 210 different mutations have been reported in the ABCB11 gene (HGMD at the Institute of Medical Genetics in Cardiff: HEXB Gene: http://www.hgmd.cf.ac.uk). Six mutations were identified in the ABCB11 gene among four patients with PFIC2, including three reported mutations (p.A588V, p.G1004D, and p.R1057X) and three novel mutations (p.R470HfsX8, p.Q794X, and p.I1170T). The missense mutation p.A588V was first reported in a compound heterozygous form with a missense mutation in a Polish patient [17]. The missense mutation p.G1004D was first reported in a compound heterozygous form with a nonsense mutation in a Chinese patient [18]. The nonsense mutation p.R1057X was first reported in a compound heterozygous form with a missense mutation in a Belgian patient [19]. The novel mutations p.R470HfsX8 and p.Q794X led to the incomplete structure of the enzyme and influenced its function. In the novel missense mutation p.I1170T, the hydrophobic isoleucine was substituted by hydrophilic neutral threonine. It was predicted to be ‘damaging’ as per the in-silico analysis using the web software. Strautnieks et al. [20] reported that p.E297G and p.D482G are the two most frequent mutations in patients of European descent, and are present in approximately 58% of European PFIC2 families included in the study. However, both these mutations were not found in our study.

ABCB4 encodes the multidrug resistance protein 3 functioning as a phospholipid floppase translocating phosphatidylcholine from the inner to the outer leaflet of the membrane [12]. So far, more than 180 different mutations have been reported in the ABCB4 gene (HGMD at the Institute of Medical Genetics in Cardiff: HEXB Gene: http://www.hgmd.cf.ac.uk). Seven mutations were identified in the ABCB4 gene among four patients with PFIC3 including one reported mutation (p.P479L) and six novel mutations (p.G319R, p.A1047P, p.G1074R, p.T830NfsX11, p.A1047PfsX8 and p.N1048TfsX). The reported mutation p.P479L had been previously reported in two patients: in homozygous form in a French patient and in compound heterozygous form with a missense mutation in a Spanish patient [21,22]. In vitro functional analysis showed that the mutation p.P479L affected the MDR3 expression level and impaired the MDR3 activity to variable degrees, clearly demonstrating its pathogenic nature [1]. In the novel missense mutations p.G319R and p.G1074R, the hydrophilic neutral glycine was substituted by hydrophilic basic arginine. These three novel missense mutations were predicted to be ‘deleterious’ as per the in-silico analysis using the web software. The other three novel variants belonged to the insertion or deletion mutations and produced truncated MDR3 protein.

In conclusion, in this study, we have reported nine Chinese patients with PFIC, including one case of PFIC1, four cases of PFIC2, and four cases of PFIC3. Fifteen different mutations were identified, including nine novel mutations (p.R470HfsX8, p.Q794X, and p.I1170T in ABCB11 gene, p.G319R, p.A1047P, p.G1074R, p.T830NfsX11, p.A1047PfsX8, and p.N1048TfsX in ABCB4 gene), and six known mutations (p.G446R and p.F529del of ATP8B1 gene, p.A588V, p.G1004D, and p.R1057X of ABCB11 gene, and p.P479L of ABCB4 gene). The results showed that compared with other regions, these three types of PFIC genes had different mutational spectrum in China. The study expands the genotypic spectrum of PFIC. We believe that the identification of nine novel mutations of PFIC and our findings could help in the diagnosis and treatment of this disease in the future.
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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Sequences of PCR primers used for the amplification of ATP8B1 gene

Click here to view

Supplementary Table 2
Sequences of PCR primers used for amplification of ABCB11 gene

Click here to view

Supplementary Table 3
Sequences of PCR primers for the amplification of ABCB4 gene

Click here to view

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