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Investigation of Common Variations of *ABCB4*, *ATP8B1* and *ABCB11* Genes in Patients with Progressive Familial Intrahepatic Cholestasis

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Abstract

Background: Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of hepatic disorders that can progress rapidly, leading to cirrhosis and death due to liver failure. Mutations and variations in three genes, including *ATP8B1*, *ABCB11*, and *ABCB4*, have been reported to be the main genetic cause of three subtypes of this disorder including PFIC1, PFIC2, and PFIC3, respectively.

Objectives: Therefore, the aim of this study was to investigate more common mutations and variations associated with PFIC considering clinical and Para-clinical features of the disease.

Methods: Thirty-five unrelated patients with PFIC from the south of Iran were selected randomly among all PFIC patients referring to Namazi hospital, affiliated to Shiraz University of Medical Sciences. Genomic DNA was extracted from the peripheral blood lymphocytes. Sequences related to these variations were then amplified by PCR in the 35 cholestasis patients and analyzed by Sanger® sequencing.

Results: The results showed that there was no variation in interest exon of *ABCB4*. Moreover, in ATP8B1, there was no prevalent mutation and only an unknown significant variation (c.*1101+366G > A) was found. However, in the ABCB11 gene, different variations were found including c.1434 + 174G > A, c.1434 + 70C > T, c.1331T > C (p.Val444Ala, a common variant proposed to be associated with cholestasis), c.1309-93G > A, c.1309-165C > T. Also, 11 and 13 cases showed heterozygote and homozygote, respectively, for V444A variation of the *ABCB11* gene.

Conclusions: The allele frequency of V444A in this study was 52.8%. This variation has been previously implicated with higher frequencies in ICP and DIC than normal subjects, suggesting that this variation may become disease-relevant in certain conditions.

Keywords: Intrahepatic Cholestasis, Mutation, *ABCB4*, *ATP8B1*, *ABCB11*

1. Background

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous class of autosomal recessive hepatic disorders that begins in the neonatal period or first year of life and usually progresses to cirrhosis within the first decade of life (1, 2). PFIC can progress rapidly and result in cirrhosis during infancy, leading to death due to liver failure at ages usually between infancy and adolescence (1, 2).

Until now, genetic and molecular studies have identified three subtypes of PFIC, which include PFIC1 (the former Byler disease), PFIC2, and PFIC3 as a result of mutations in genes involved in bile formation. PFIC1 is due to *ATP8B1* gene mutations, which can cause the milder phenotype (3). PFIC2 is caused by mutations in the *ABCB11* gene (4, 5). This gene which is located on human chromosome 2q24 encodes the ATP-dependent canalicular bile salt export pump (BSEP) in liver and the loss of BSEP function responsible for the decreased biliary bile salt secretion resulting in the accumulation of bile salts inside the hepatocyte and finally severe hepatocellular cholestasis (6). PFIC3 results from diseases causing variants in the *ABCB4* gene located on chromosome 7q21. This gene is translated into the protein MDR3 with an essential role in biliary phospholipid (phosphatidylcholine) excretion across the canalicular membrane (7-9). Bile from patients with PFIC3 is not inactivated by phospholipids (with very low concentrations of phospholipid) that results in bile canaliculi and biliary epithelium injuries, leading to cholangitis and cholestasis cholestatic liver disease (10). Given the fact that different *ATP8B1*, *ABCB11*, and *ABCB4* variants have been associated with cholestasis, we made an attempt to investigate the

most common variants reported in these genes among 35 patients with cholestasis.

2. Methods

2.1. Patients

Thirty-five unrelated patients with cholestasis from the south of Iran were enrolled in this study. All cholestasis patients were diagnosed by a group of gastroenterohepatology specialists in gastroenterohepatology ward of Namazi hospital, affiliated to Shiraz University of Medical Sciences, on the basis of characteristic symptoms such as jaundice, dark urine, light-colored stools, and generalized itchiness. The patients were selected randomly. All the patients gave informed consent before undergoing genetic analysis for common variations of *ATP8B1*, *ABCB4*, and *ABCB11*. Three-milliliter whole-blood samples were collected from the patients, drawn into EDTA tubes, and stored at -20°C until use.

2.2. Preparation of Genomic DNA

Genomic DNA was extracted from the peripheral blood lymphocytes by QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The genomic DNA concentration was measured by NanoDrop (ND1000, USA) and stored at - 20°C until use.

2.3. PCR and Sequencing

Sequences covering the variations of ATP8B1 (I661T, exon 18), ABCB4 (p.R652G, exon 16), and ABCB11 (p.V444A, exon 13) in 35 cholestasis patients were amplified by PCR primers given in Table 1. These primer pairs were designed and evaluated on the basis of the *ATP8B1*, *ABCB4*, and *ABCB11* reference genomic sequences (ENSEMBLE) using NCBI-BLAST, UCSC (BLAT and In Silico PCR) and Allele ID 7.5 (Table 1). The total volume of the PCR was 50 μL containing 1 μL of each primer (20 pmol/μL), 1 μL DNA template (50 - 200 ng), 25 μL TEMPase Hot Start 2x Master Mix Blue (Ampicon, A290806), and 22 μL dH_2O. The PCRs were carried out according to Amplicon TEMPase Hot Start protocol and programs given in Table 1. Ten microliters of the PCR products were visualized on 2% agarose gel containing SYBR Safe.

2.4. DNA Sequencing

PCR products of interest exons and exon-intron boundaries of three mentioned genes in our 35 patients were analyzed by DNA sequencing. Sanger sequencing data were analyzed using NCBI BLAST and Codon Code Aligner software.

3. Results

A total of 35 patients (70 alleles) with progressive intrahepatic cholestasis were studied for detection of common variants associated with this disorder in interest exons of *ATP8B1* (I661T), *ABCB4* (p.R652G), and *ABCB11* (p.V444A). There was not identified any variation in interest exon of *ABCB4*. Also, in *ATP8B1*, a prevalent mutation was not identified, and only an unknown significant variation (c.*1101 + 366G > A) was identified in patients 4, 9, 15, 21, 26, and 29 as heterozygote and patient 14 as homozygote. However, in ABCB11 gene, different variations were found including c.1434 + 174G > A, c.1434 + 70C > T, c.1331T > C (p.Val444Ala, common variant), c.1309-93G > A, and c.1309-165C > T. Since among these variations, only p.Val444Ala is important due to its susceptibility and association with intrahepatic cholestasis, its distribution in our patients are given in Table 2.

It was found that there were 11 and 13 cases of heterozygote and homozygote, respectively, for V444A variation of *ABCB11* gene. As mentioned above, this change results in a reduction in the quantity of BSEP protein in liver cells and may be associated with cholestasis in certain conditions, such as pregnancy and the use of ethinylestradiol and levonorgestrel. Among 35 patients involved in this study, 10 cases showed no mutation or variation in the investigated exons of the three genes and other exons should be investigated for their genetic origin. All data are given in Table 2.

Clinical presentation and signs observed in our patients were jaundice, itching, bleeding tendency, fever, tea-color urine, pruritus, ascites, gastrointestinal bleeding, encephalopathy, and itching wounds. Their Para clinical findings on the basis of ultrasonography and histopathological examination indicated enlarged liver, inhomogeneous liver echo, spleen prominent size ascites pleural effusion, cirrhosis, chronic cholecystitis, fibrosis, splenomegaly, hepatomegaly, giant cell formation, and intracalcinular cholestasis (Table 2).

4. Discussion

Molecular genetic testing to identify mutations and variations associated with cholestasis in *ATP8B1*, *ABCB4*, and *ABCB11* genes is important to confirm PFIC2 diagnosis. Different mutations in these genes have been identified in Asian population (11, 12). The majority of them are different from those identified in other populations. Prevalent mutations that have been reported in Europe were not detected in other regions such as China. Therefore, mutations in these genes may be ethnicity-specific (13)

Table 1. Primers and PCR Protocols Used for Amplification of the Interest Exons of *ATP8B1* (NI661T), *ABCB4* (R652G) and, *ABCB11* (V444A) Genes

Gene (Interest Variant)	Primer Sequence	PCR Program	PCR Product
ATP8B1 **(NI661T)**	Forward: GGATGATAAAGCCAGACCTTGT	95°C for 15 min, 35 cycles for: 95°C, 30 sec, 64°C, 30 sec, 72°C, 20 sec, and final extension 72°C, 7 min	502 bp
	Reverse: GTGCCAGTGTCAAATGCTGAA		
ABCB4 **(R652G)**	Forward: TCCTTGATTGAGAAGCAGTTAG	95°C, 15 min, 35 cycles for: 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, and final extension 72°C for 7 min	571 bp
	Reverse: GCATCTCAGCGTAAAGACTAC		
ABCB11 **(V444A)**	Forward: TCTTGGTCATGGCTCTCA	Touch Down PCR, 95°C for 15 min, 20 cycles for: 95°C for 30 sec, 67°C for 30 sec (-0.5°C per cycle), 72°C for 20 sec, 15 cycles for:95°C 30 sec, 58°C 30 sec, 72°C for 25 sec, and final extension 72°C for 7 min	626 bp
	Reverse: ATCACTGACTGAAATGTTGC		

V444A is a highly prevalent variant of ABCB11 and its allele frequency has been reported in Japanese and Caucasian populations (14). The allele frequency of V444A in the current study was 52.8%. This variation has been previously implicated with higher frequencies in ICP and DIC than normal subjects, suggesting that this variation may become disease relevant in certain conditions (15, 16). However, further larger-scale studies are required to fully uncover the role of V444A variant.

Bile salt export pump (BSEP) is highly conserved during vertebrate evolution, and its expression is inhibited by PFIC type II mutations (17). PFIC type 2 is due to mutations in ABCB11, the gene encoding the BSEP protein. In our study, most of the patients (62.85%) had homozygote mutations in *ABCB11* gene. Jaundice, itching, bleeding tendency, fever, tea-color urine, enlarged liver, normal echo, intracellular and intracalcinular cholestasis, cholestasis, pruritus, ascites, GI bleeding, encephalopathy, increased liver echo, cholestasis, giant cell formation fibrosis, and cirrhosis were some of the clinical and Para clinical features of the PFICII in our patients.

ATP8B1 deficiency is a severe autosomal recessive liver disease resulting from mutations in the *ATP8B1* gene characterized by a spectrum from intermittent (benign recurrent intrahepatic cholestasis; BRIC) to progressive familial intrahepatic cholestasis (PFIC) (18).

About 20% of our cases had homozygote and heterozygote mutations in their *ATP8B1* genes. However, most of them had also mutations in their *ABCB11* gene. Jaundice, poor growth, pruritus, encephalopathy, ascites, GI bleeding, enlarged liver, coarse echo, chronic hepatic parenchymal damage, focal steatosis and ballooning degeneration, fibrosis as well as elevated liver enzymes, heterogeneous echo, relative destruction of lobular and vascular architecture, fibrosis, splenomegaly, hepatomegaly, relative destruction of lobular and vascular architecture were clinical and Para clinical findings of the disease in our patients confirmed by the detection of the mutations.

Different molecular methods have been used to investigate mutations in genes associated with cholestasis such as single-strand conformation polymorphisms (SSCP) (19), denaturing high performance liquid chromatography (DHPLC), and DNA sequencing (20). The gold standard is DNA sequencing since it can reveal mutations and variations across three genes with 28 exons for each. However, this method is very expensive. Therefore, the current study tried to investigate common mutations and variations associated with cholestasis in three specific genes and their main exons.

Our study showed that while two common mutations were not identified in our patients, the other common variant, V444A, that previously has been proposed to be associated with cholestasis in certain condition such as pregnancy and the use of ethinylestradiol and levonorgestrel, was identified in a high frequency (37 chromosomes out of 70, 52.8%). The common V444A variant can be investigated in control samples to be concluded for its susceptibility and association with intrahepatic cholestasis in our patients since this variant leads to a reduction in the quantity of BSEP protein in liver cells and can be a good indicator in these patients (15).

In conclusion, the current study investigated common

mutations and variations of *ATP8B1*, *ABCB4*, *ABCB11* genes. V444A was a highly prevalent variation found in *ABCB11* in the study. However, it is not fully clear that if they are associated with pediatric cholestatic diseases. More studies should be conducted to investigate this variation, identify the molecular mechanism of *ABCB11* product and BSEP and therefore, determine its usefulness in the personalized management of special patients.

There are so many studies that have evaluated the variations in *ABCB4*, *ABCB11* and *ATP8B1* genes separately. However, no study investigated the mutations of three genes in each study patient. This is considerable because we found that a large number of our cases (17.14%) had mutations in both *ABCB11* and *ATP8B1* genes.

One of the limitations of the study was to find patients who voluntarily agreed to participate in this study and donate blood samples. On the other hand, more developed sequencing of related genes, for example whole exon sequencing, was needed to find underlying mutations in cases with no detected mutation using sanger sequencing. In our study, some of the patients had no mutation in interest areas of three mentioned genes, so they should be evaluated by further molecular investigations to find molecular basis of the disease.

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Footnote

Conflict of Interest: None declared.

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Table 2. Clinical and Para Clinical Sings of the Patients and Distribution of c.1331T > C (p.Val444Ala) in *ABCB11* Gene among Our Patients

Case No.	ABCB11	ABCB4	ATP8B1	Clinical Signs	Para Clinical Findings	Treatment
1.		1	ND	Jaundice, Itching, Bleeding Tendency, Fever, Tea Color Urine	Enlarged Liver, Normal Echo, Intracellular and Intracalcinular Cholestasis, Fibrosis	LT
2.	Hom.	ND	ND	pruritus, Ascites, GI bleeding, encephalopathy	increased liver echo, cholestasis, giant cell formation, cirrhosis	LT
3.	ND	ND	ND	Pruritus, clay stool, itching, jaundice, hepatomegaly, irritability	heterogeneous echo of the liver, enlarged spleen, destruction of lobular and vascular architecture, nodule formation, cirrhosis	LT
4.	Het.	ND	Het.	poor growth, pruritus, encephalopathy, ascites, GI bleeding,	enlarged liver, coarse echo, chronic hepatic parenchymal damage, focal steatosis and ballooning degeneration, fibrosis	LT
5.	Hom.	ND	ND	Jaundice	increased echo, enlarged liver, enlarged spleen, cirrhosis	LT
6.	Het.	ND	ND	pruritus, vomiting, diarrhea, fatty stool, tea color urine hepatomegaly, itching	enlarged liver, enlarged spleen, fibrosis, cirrhosis	LT
7.	Hom.	ND	ND	jaundice, icteric sclera, itching	liver coarse echo	LT
8.	Het.	ND	ND	Jaundice, pruritus, encephalopathy	liver prominent size, enlarged spleen, cirrhosis, giant cell hepatitis	LT
9.	Het.	ND	Het.	Jaundice	Elevated liver enzymes, heterogeneous echo, relative destruction of lobular and vascular architecture, fibrosis	LT
10.	Hom.	ND	ND	Jaundice	Elevated liver enzymes, splenomegaly, hepatomegaly, hyperechoic lesion, spotty necrosis, infiltration of lymphocytes, fibrosis	LT
11.	ND	ND	ND	Jaundice	enlarged liver, increased echo of hepatic biliary tree, infiltration of inflammatory cells, fibrosis	LT
12.	Hom.	ND	ND	Jaundice, Yellowish Skin, encephalopathy, GI bleeding, Ascites, Pruritus	enlarged spleen, inhomogeneous echo, cirrhosis	LT
13.	Hom.	ND	ND	Pruritus	mucosal thickening of Gall bladder, Ascites, fibrosis	LT
14.	Hom.	ND	Hom.	Pruritus, Pruritus Yellow Skin, And Sclera, Itching Wound	Altered liver enzymes	LT
15.	Hom.	ND	Hom.	Pruritus, Ascites Jaundice, Encephalopathy	irregular outline of liver, coarse echo, spleen prominent size ascites pleural effusion, Cirrhosis, chronic cholecystitis	LT
16.	ND	ND	ND	No data was available	No data was available	-
17.	Hom.	ND	ND	Jaundice	enlarged liver, inhomogeneous echo	LT
18.	Het.	ND	ND	Itching, Jaundice, anorexia, pruritus encephalopathy, GI bleeding	enlarged liver, Gall stone, duct proliferation, inflammation, Rosette formation, Feathery changes	LT
19.	ND	ND	ND	Itching, intractable pruritus	Altered liver enzymes	LT
20.	ND	ND	ND	Itching, severe pruritus	Altered liver enzymes, increased liver echo	LT
21.	Hom.	ND	Het.	Jaundice, icterus	liver prominent size, hyperechoic liver, spleen prominent size, Extensive Fibrosis, portal fibrosis, ductular proliferation, cholestasis	Liver biopsy
22.	Hom.	ND	ND	Jaundice, icterus	Elevated liver enzymes, Liver coarse echo, enlarged spleen, destruction of lobular and vascular architecture, nodule formation, fibrosis	LT
23.	Hom.	ND	ND	Jaundice, pruritus, encephalopathy, ascites, GI bleeding, palmar erythema, GI bleeding	patent spleen, giant cell formation, feathery degeneration, fibrosis	LT
24.	ND	ND	ND	Itching, Jaundice, generalized pruritus	enlarged liver with homogenous echo	BD, LT
25.	ND	ND	ND	Itching, pruritus	cholestatic rosettes, fibrosis	LT

26.	ND	ND	Het.	Altered liver enzymes	irregular capsule, coarse echo, multifocal dilation of biliary ducts, cirrhosis	LT
27.	Het.	ND	ND	Itching, Jaundice, fever, tea color urine, GI bleeding, Encephalopathy	Enlarged Liver	LT
28.	Het.	ND	ND	Itching, Jaundice, icteric, sever pruritus, irritability, jaundice, ecchymosis, bleeding tendency	enlarged liver, inhomogeneous echo	LT
29.	Het.	ND	Het.	Jaundice, bleeding tendency,	splenomegaly, hepatomegaly, relative destruction of lobular and vascular architecture, fibrosis	LT
30.	ND	ND	ND	Jaundice, yellowish skin, pruritus	enlarged liver, coarse echo	LT
31.	Het.	ND	ND	prolonged jaundice	cholestatic rosettes, fibrosis	LT
32.	Hom.	ND	ND	jaundice	hepatomegaly, elevated LFT	LT
33.	ND	ND	ND	No data was available	No data was available	-
34.	ND	ND	ND	No data was available	No data was available	-
35.	Het.	ND	ND	sever itching, encephalopathy, ascites, GI bleeding	Cirrhosis, increased echo, enlarged spleen	LT

Abbreviations: BD: Biliary diversion; LT, Liver transplantation; Het, heterozygote; Hom, homozygote; ND, Non-detected.