

ORIGINAL ARTICLE

DHPLC screening for mutations in progressive familial intrahepatic cholestasis patients

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Progressive familial intrahepatic cholestasis (PFIC) is a group of rare heterogeneous autosomal recessive disorders characterized by metabolic defects in biliary proteins involved in the formation and transfer of bile acids in the liver. The genotype–phenotype correlation is not always clear. Mutations in the *ATP8B1*, *BSEP* and *MDR3* genes have been associated with PFIC1, PFIC2 and PFIC3, respectively. This study sought to characterize the molecular genetic basis for PFIC subtypes in Israel. It was conducted on 14 children with PFIC and their families; 10 with a PFIC1 or PFIC2 phenotype and 4 with a PFIC3 phenotype. Using denaturing high-performance liquid chromatography (DHPLC), five different mutations were identified in four affected families: three novel mutations in *BSEP* (G19R-g181c, S226L-c803t and G877R-g2755a), one novel mutation in *MDR3* (IVS14+6 t/c) and one heterozygous mutation in *ATP8B1* (R600W, in a family with the PFIC1/PFIC2 phenotype). The cause of PFIC was identified in 20% of the families tested. These findings indicate the probable involvement of additional genes in PFIC and the need for further studies to determine whether the abnormality lies on the RNA or protein level. A better understanding of the phenotype–genotype correlation in PFIC will lead to improved diagnoses and treatments.

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INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a group of rare genetic heterogeneous autosomal recessive disorders characterized by defects in the biliary proteins involved in the formation and transfer of bile salts in the liver.^{1,2} The disease becomes evident in infancy, and without suitable treatment, may lead to death in the first decade of life.³ It is divided into three subtypes. The PFIC1 phenotype is caused by a variety of mutations in the *ATP8B1* gene, which encodes for P-type adenosine triphosphatase (ATPase), a protein expressed in liver parenchymal cells and several organs.¹ The protein probably functions as an aminophospholipid translocase and may have a role in the circulation of bile acids.² PFIC2 is caused by mutations in the bile salt export pump (*BSEP*) gene (or *ABCB11*), which encodes for an ATP-binding cassette (ABC) transporter protein that is expressed only in the canalicular membrane of the biliary tree and transfers bile salts from the liver to the gall bladder.⁴ PFIC3 is caused by mutations in the *MDR3* gene (or *ABCB4*), which encodes for multidrug resistance P-glycoprotein,³ which is involved in the availability of phospholipids in the canalicular bile that help to organize the canalicular and protect them from toxicity due to a high bile salt concentration.⁵ Defects in

MDR3, but not in *ATP8B1* or *BSEP*, are associated with high levels of serum γ -glutamyltranspeptidase.⁴

The genotype–phenotype correlation in PFIC1 and PFIC2 is not always clear because of their considerable clinical overlap.⁶ Therefore, DNA screening for mutations in *ATP8B1* and *BSEP* is usually necessary for diagnosis.³ The definitive treatment for PFIC is liver transplantation.⁷ Pharmacological treatments for the associated liver disease include (1) simvastatin (used in PFIC1), a hydroxymethyl glutamyl coenzyme A reductase inhibitor, which reduces the synthesis of bile acid from its precursor, cholesterol 2 and (2) ursodeoxycholic acid (used in PFIC3), which exerts a protective inhibitory effect on hepatocyte apoptosis by effecting a change in the arrangement of bile acid synthesis.⁸

The aims of this study were to characterize the molecular genetic basis of PFIC subtypes in Israel. We sought to identify mutations in the genes associated with the PFIC phenotypes and to screen for two additional candidate genes, farnesoid X receptor (*FXR*) and Na⁺-dependent taurocholic co-transporting polypeptide (*NTCP*). The *FXR* gene, which encodes for a nuclear receptor involved in the regulation of *BSEP* and *MDR3*,⁹ is expressed in all tissues in which bile

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salts are present.¹⁰ *NTCP* encodes for a transporter of bile salts to the liver.³ Both have been found to be expressed differently in patients with PFIC when compared with that in healthy controls.⁹

MATERIALS AND METHODS

Study group

The study was conducted on 14 PFIC children and their families (including first-degree family members); 10 children with a PFIC1 or PFIC2 phenotype and 4 with a PFIC3 phenotype. All people participating in the study had given their informed consent before their inclusion in the study. Family histories were obtained by questioning the families/parents regarding any previous cases of neonatal jaundice, chronic liver diseases or liver transplantation in siblings. Of the 14 families, 11 were of Arabic (10 Muslim and one Druze) origin, and three were Jewish. All patients of Muslim origin were the outcome of consanguineous marriages, with parents being either first or second cousins. Among the Jewish patients, who were Ashkenazi (families 8, 12 and 14), no consanguinity was found. The patients' backgrounds and laboratory findings are shown in Table 1.

This retrospective study uses the files of these 14 children who during the years 1998–2007 had been examined for chronic cholestasis, with elevated or normal γ -glutamyltranspeptidase. In the present investigation, subjects with infectious, autoimmune or metabolic diseases were ruled out. In all but two of the patients, a liver biopsy had been performed, which did not contribute to the diagnosis.

DNA extraction and PCR amplification

DNA was extracted from peripheral blood leukocytes according to standard protocols. All coding regions of the *ATP8B1*, *BSEP* and *MDR3* genes and the intron/exon boundaries were amplified using sense and antisense oligonucleotides that were designed and numbered on the basis of the known sequence of the gene loci (GenBank accession numbers: NM_005603.3, NM_003742.2 and NM_018849.2, respectively). Amplifications were carried out by PCR in a total volume of 50 μ l with 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM deoxynucleotide triphosphates and 30 nM primers. The PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing temperatures for 1 min and 72 °C for 1 min, as described previously.¹¹ The reactions were concluded with a final extension step of 72 °C for 7 min. The primers, annealing temperatures and sizes of the PCR products for each of the 27 exons of *ATP8B1*, *BSEP* and *MDR3* are available from the authors upon

request. Patients with the PFIC1 or PFIC2 phenotypes were screened for *ATP8B1* and *BSEP*, and patients with the PFIC3 phenotype were examined for mutations in *MDR3*. When no mutations were identified, the coding regions of the two other candidate genes, *FXR* (9 exons) and *NTCP* (5 exons) (GenBank accession numbers: NM_005123.2 and NM_003049.2, respectively), were screened.

Semiquantitative PCR

Reactions were carried out in a total volume of 50 μ l with 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM deoxynucleotide triphosphates and 30 nM primers. The concentration of the DNA samples (from the suspected patient, and his mother as the control) was 50 ng μ l⁻¹. The PCR conditions¹¹ were 94 °C for 5 min, followed by 24 cycles of 94 °C for 0.5 min and annealing temperatures for 1 min and 72 °C for 1 min. A final extension step of 72 °C for 7 min ended the PCR in the log stage. The exons assayed were exon 21 (359 bp) (5'-GTGTAATGGTAATTGGTAAAAGCG-3' and 5'-CCTTCAGTCTCTTCGTA CTACTCG-3') in the *BSEP* gene and exon 14 (324 bp) (5'-CTAAAATCCTA AGGTGGTTTTTGC-3' and 5'-ACAAAAAACCTGGTCTCTAATGCC-3') in the *ATP8B1* gene, which served as the control.

Denaturing high-performance liquid chromatography (DHPLC) mutation screening

To scan for DNA mutations and variants using DHPLC, PCR products were subjected to chromatography using an ion-pair reversed-phase cartridge. The products were denatured and allowed to reanneal. Under conditions of partial denaturation with a linear acetonitrile gradient, heteroduplexes from PCR samples, which have an internal sequence variation, showed a reduced column retention time relative to their homoduplex counterparts. Because the elution profile for heterozygous samples is typically quite distinct from that of both homozygous sequences, the identification of heterozygous mutations was relatively straightforward.¹¹ As required in homozygous autosomal mutations or in mutations on the X chromosome in males, the test sample was mixed with DNA of a known sequence.¹²

Establishing DHPLC conditions

Mutation analysis was performed using the WAVE apparatus (Transgenomic, Omaha, NE, USA). The PCR products were denatured at 95 °C for 5 min and cooled to 65 °C, along a decreasing temperature gradient of 1 °C min⁻¹. The

Table 1 Characterization of PFIC phenotypes

Family no.	Sample no.	Sex	Age at diagnosis (years)	Consanguinity	GGT	LBx	LTx	Outcome
1	1	M	4	Y	17	Y	N	MR, FTT, CLD
2	3	M	0.8	Y	29	Y	N	MR, FTT, CLD
3	5	M	3	Y	27	Y	Y	A&W
3	7	M	5	Y	17	Y	Y	A&W
4	10	M	0.16	N	32	Y	N	A, CLD
5	13	M	0.3	N	21	N	N	A, BD, FTT
5	14	M	0.16	Y	20	Y	Y	A&W
6	17	M	0.3	N	25	N	N	A, CLD, severe MR
7	31	F	0.1	N	24	Y	N	BD, healthy
7	32	F	0.05	N	28	Y	N	BD, mild choles
8	34	M	0.08	Y	36	Y	Y	A&W
9	41	F	14	N	6	Y	N	A&W
10	46	—	—	—	—	—	—	—
11	20	M	0.75	Y	520	Y	YX4	Death
12	23	F	—	—	—	N	—	—
13	28	F	0.2	N	1456	Y	N	BD, choles
14	36	M	1	N	156	Y	N	Death, HCC
14	39	M	0.41	N	107	Y	N	A and CLD

Abbreviations: A, alive; A&W, alive and well; BD, biliary diversion; Choles, cholestasis; CLD, chronic liver disease; F, female; FTT, failure to thrive; GGT, γ -glutamyl transferase; HCC, hepatocellular carcinoma; LBx, liver biopsy; LTx, liver transplantation; M, male; MR, mental retardation; N, no; PFIC, progressive familial intrahepatic cholestasis; Y, yes.

samples were kept at 4 °C until 5 µl was applied to a preheated C18 reversed-phase column, based on nonporous particles (polystyrene-divinyl-benzene) (DNA-Sep Cartridge, cat. no. 450181; Transgenomic). DNA was eluted within a linear acetonitrile gradient, consisting of buffer A (0.1 M triethylammonium acetate, cat. no. SP5890; Transgenomic)/buffer B (0.1 M triethylammonium acetate, 25% acetonitrile, cat. no. 700001; Transgenomic)]. The temperature at which heteroduplex detection occurred was deduced by the Transgenomic software (Wavemaker 4.2) and the Stanford DHPLC melting program (<http://insertion.stanford.edu/meltdoc.html>), which analyzes the melting profile of each specific DNA fragment.

Linkage analysis

The single-nucleotide polymorphisms (SNPs) that were selected for linkage analysis were established SNPs (HapMap and NCBI (National Center for Biotechnology Information) database, <http://www.ncbi.nlm.nih.gov/>), which have high heterozygote frequencies (<30%) in the relevant ethnic group. Primers were designed accordingly. According to SNPs of DNA samples from patients and their parents, if available, the allelic pattern inherited in the family was defined to determine whether this genomic region was involved in these phenotypes. The SNP NCBI association numbers are as follows: for *BSEP*—rs3814381, rs478333, rs3770598, rs6759156, rs2892809, rs555975, rs503931, rs2685814, rs2241339, rs2058996, rs1101533 and rs853779; for *MDR3*—rs1526090, rs12154399, rs4148830, rs31667, rs31670, rs31674, rs31676, rs1558376, rs4148816 and rs4148809; and for *FXR*—rs12297839, rs7304328, rs2148670, rs2303624, rs11110414, rs10860603, rs35735, rs11110411 and rs7960489.

Homozygous mutation screening

To identify homozygous mutations, 10 µl of each PCR product from wild-type DNA and 10 µl of the PCR product of sample DNA were mixed 1:1 and denatured at 95 °C. The formation of heteroduplexes indicated homozygosity.¹²

DNA sequencing

DNA sequencing was performed on the PCR products after DHPLC analysis, as previously described.¹¹ Reamplified PCR products (forward and reverse) with an abnormal DHPLC pattern were subjected to sequence analysis by an ABI PRISM 3100 Genetic analyzer (PE Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Nucleotide numbering was in accordance with the GenBank database of nucleotide sequences (accession numbers NM_005603.3, NM_003742.2, NM_018849.2, NM_005123.2 and NM_005123.2).

RESULTS

The findings of the genetic analysis are shown in Table 2. For the first-line assessment, the entire coding region of *ATP8B1*, the first gene discovered to be involved in PFIC1,¹³ was screened. From this, only one heterozygous mutation (R600W) was identified, and it was in family.⁹ This mutation is known to have a role in benign recurrent intrahepatic cholestasis.¹⁴

Subsequently, linkage analysis was conducted on the *BSEP* gene. The SNP haplotype (TTCC) was found in two patients from family 3, but not in their healthy sibling (Figure 1), implying a DNA alteration in the region related to the PFIC2 phenotype. Screening this family's DNA yielded two novel heterozygous mutations in *BSEP* (G19R-g181c and S226L-c803t). Each of the mutant alleles was inherited from one of the parents (that is, the children were probably compound heterozygotes; Figure 2).

In other families in which the linkage study was not informative, the entire gene was screened. Family 2 was found to harbor a novel mutation, G877R-g2755a, in a homozygous state (Figure 3). The mother of this family was a carrier, and the paternal DNA was not available for analysis. As the patient was found to be homozygous for all SNPs in the screened gene, semiquantitative PCR analysis was used in an attempt to determine whether the mutation was

Table 2 Results from the screening of the five candidate genes

Family	DNA	<i>ATP8B1</i>	<i>BSEP</i>	<i>MDR3</i>	<i>FXR</i>	<i>NTCP</i>
1	1-patient	WT	WT	—	WT	WT
	1-M	WT	WT	—	WT	WT
2	2-patient	WT	G877R	—	WT	WT
	2-M	WT	G877R/N	—	WT	WT
3	3-patient	WT	G19R/N; S226L/N	—	WT	WT
	3-B	WT	S226L/N	—	WT	WT
	3-patient	WT	G19R/N; S226L/N	—	WT	WT
	3-M	WT	S226L/N	—	WT	WT
	3-F	WT	G19R/N	—	WT	WT
4	4-patient	WT	WT	—	WT	WT
	4-M	WT	WT	—	WT	WT
	4-B	WT	WT	—	WT	WT
5	5-patient	WT	WT	—	WT	WT
	5-patient	WT	WT	—	WT	WT
	5-M	WT	WT	—	WT	WT
	5-F	WT	WT	—	WT	WT
6	6-patient	WT	WT	—	WT	WT
	6-M	WT	WT	—	WT	WT
	6-F	WT	WT	—	WT	WT
7	7-patient	WT	WT	—	WT	WT
	7-patient	WT	WT	—	WT	WT
	7-M	WT	WT	—	WT	WT
8	8-patient	WT	WT	—	WT	WT
	8-M	WT	WT	—	WT	WT
9	9-patient	R600W/N	WT	—	WT	WT
	9-F	WT	WT	—	WT	WT
	9-M	R600W/N	WT	—	WT	WT
	9-S	R600W/N	WT	—	WT	WT
10	9-S	WT	WT	—	WT	WT
	10-patient	WT	WT	—	WT	WT
11	10-F	WT	WT	—	WT	WT
	11-patient	—	—	WT	WT	—
	11-M	—	—	WT	WT	—
12	11-F	—	—	WT	WT	—
	12-patient	—	—	IVS14+6t/c/N	WT	—
13	12-F	—	—	WT	WT	—
	13-patient	—	—	WT	WT	—
	13-M	—	—	WT	WT	—
14	13-F	—	—	WT	WT	—
	14-patient	—	—	WT	WT	—
	14-M	—	—	WT	WT	—
	14-F	—	—	WT	WT	—
	14-B	—	—	WT	WT	—
	14-S	—	—	WT	WT	—

Abbreviations: B, brother; *BSEP*, bile salt export pump; F, father; *FXR*, farnesoid X receptor; M, mother; *NTCP*, Na⁺-dependent taurocholic co-transporting polypeptide; S, sister; WT, wild type.

hemizygous (that is, one mutated allele and one deleted allele). Results of this analysis, which compared the relative quantity of the *BSEP* alleles in the maternal and patient DNA (Figures 4a and b), revealed that the mutation was present in the hemizygous state in the patient.

Linkage analysis was performed on DNA from three of the four families with PFIC3 (family 12 was not tested because the maternal DNA was unavailable). No association with the *MDR3* gene was found in family 14, but its involvement in families 11 and 13 could not be ruled out. In addition, DNA screening for *MDR3* mutations in families 11, 12 and 13 yielded one heterozygous novel splice-site

mutation (IVS14+6 t/c) in the patient from family 12. The origin of the mutation could not be proven, as only DNA from the father, who did not harbor the mutation, was available. No other mutations were

identified in the remaining coding and promoter regions of the *MDR3* gene in this patient.

Linkage analysis and mutation screening of the two suggested candidate genes, *FXR* and *NTCP*, produced only negative results.

In total, five mutations were discovered in the *ATP8B*, *BSEP* and *MDR3* genes; that is, three novel mutations in *BSEP* (G19R-g181c, S226L-c803t and G877R-g2755a), one novel mutation in *MDR3* (IVS14+6 t/c) and only one heterozygous mutation in *ATP8B1* (R600W).

DISCUSSION

In this study, the genetic cause of PFIC1/PFIC2 was identified in 2 of 14 children (10 families). The patients from family 3 were compound heterozygotes for two mutations in *BSEP*—G19R and S226L—and the patient from family 2 had a hemizygous mutation in *BSEP*—G877R. S226L and G877R are missense mutations at the conserved and active sites of the proteins (ConSeq; <http://conseq.tau.ac.il>; Figure 5), and G19R is located at the N-terminal region of the BSEP protein. In another family with PFIC1/PFIC2 (family 9), one missense mutation was found in *ATP8B1* (R600W). However, it could not account for the phenotype. The same mutation has been reported previously in a patient with benign recurrent intrahepatic cholestasis.¹⁵ However, unlike PFIC, benign recurrent intrahepatic cholestasis has a mild phenotype, as the *ATP8B1* mutations associated with the disease do not have a deleterious effect on protein function.

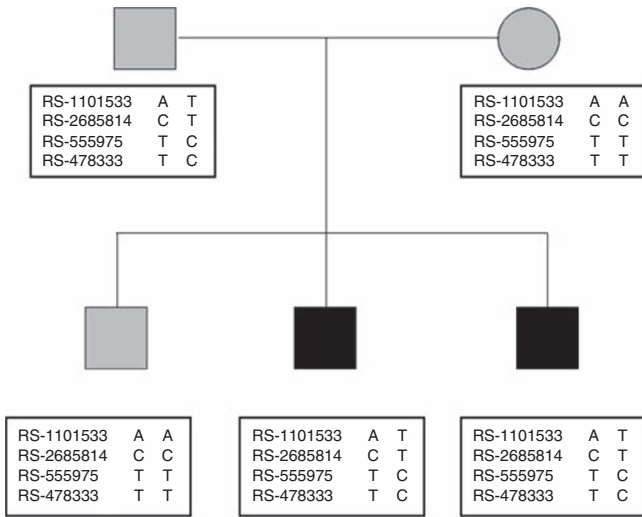


Figure 1 Structure of allele segregation in family 3 according to SNPs in *BSEP* gene. Black, patient; gray, carrier. SNP genotypes appear in the boxes.

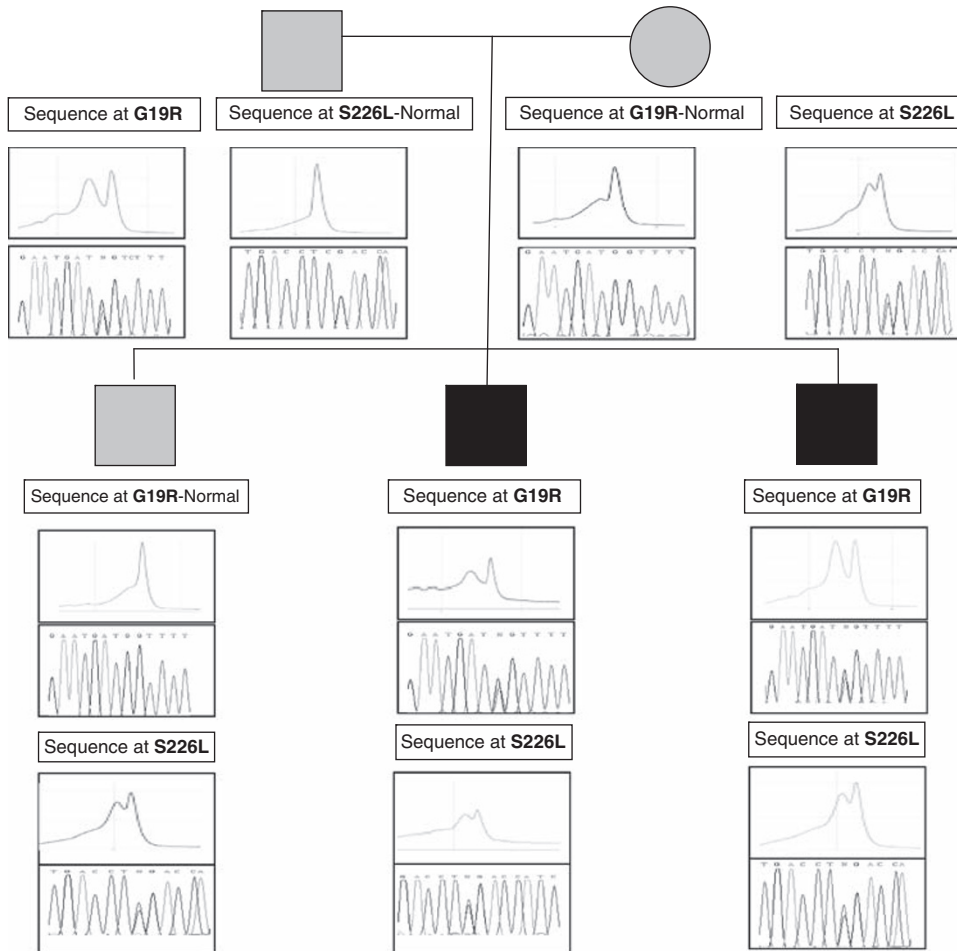


Figure 2 DHPLC analysis and sequence of mutations in family 3. There are two different heterozygous mutations in the *BSEP* gene. The patients inherited each mutant allele from a different parent, black, patient; gray, carrier. Mutations detected by the DHPLC are shown in the upper figure and their sequences are in the lower figure.

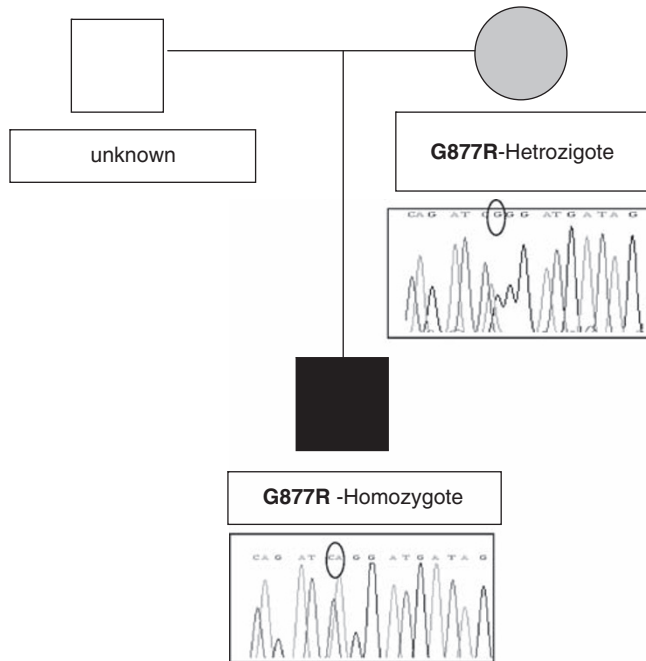


Figure 3 Sequence analysis of the G877R (2755g>a) missense mutations of the *BSEP* gene in family 2. Only the DNA samples of the female patient and her healthy mother were available for screening. The mother was found to be heterozygous and the daughter hemizygous for this mutation.

These mutations were compared with those discovered in other populations, but four out of five were not found. The mutation R600W was sited in one rare case (adult patient) in Taiwan. All of these families (2, 3 and 9) were of Muslim origin, and the patients were the result of consanguineous marriages, with parents being either first or second cousins. As these mutations have not been reported previously, they could not be compared with other ethnic groups.

In family 12, with the PFIC3 phenotype, one heterozygous mutation was discovered on the *MDR3* gene of the patient. However, alone, it could not be the reason for the disease. It is possible that there is another heterozygous mutation in a control region that was not discerned. None of these five mutations were detected in the screening of 200 alleles in DNA control samples.

The screening of the two candidate genes, *FXR* and *NTCP*, known to be involved in bile acid homeostasis in the liver 7, yielded no mutations.

PFIC is a heterogeneous disease, and it is likely that other unknown genes are involved. Clinical criteria alone are not enough to identify the molecular defects in PFIC2, and genetic screening is the best method to differentiate PFIC2 from PFIC1 4. Because of its grave prognosis 3, genetic counseling and prenatal diagnosis are recommended in affected families.

In the present study, by combining the clinical diagnosis of PFIC with the screening of suspected genes by DNA chromatography and direct sequencing, we were able to identify the causes of the disease in 20% of the families tested. All analyses were carried out on the DNA level, as liver biopsies were not available. Further studies on the RNA and protein level are required to determine whether the abnormalities lie in gene expression or gene translation.

Our results point out the heterogeneity of PFIC, and hopefully the new genetic technology that has emerged recently will help to elucidate the 80% unsolved cases. For example, homozygosis mapping

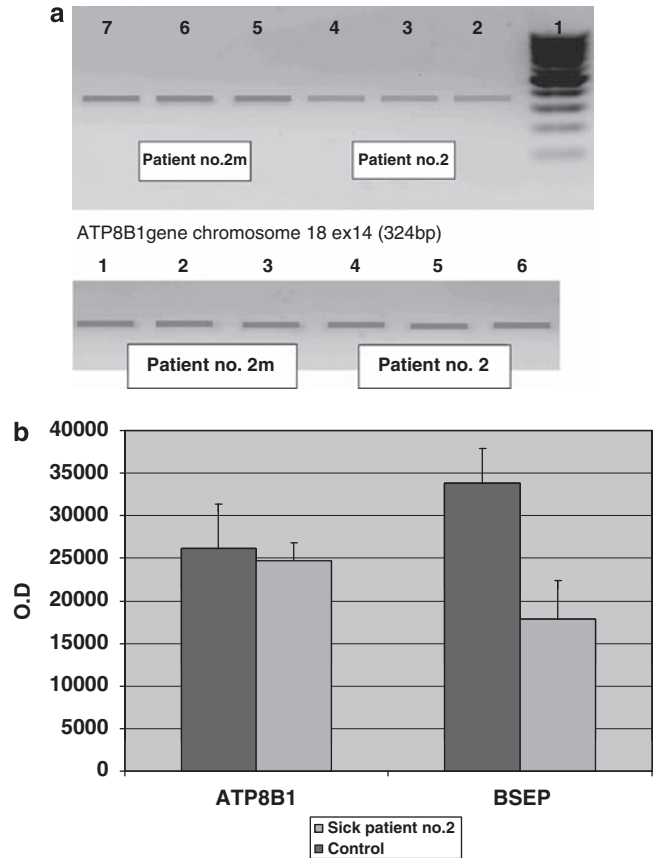


Figure 4 The semiquantitative PCR analysis of the *BSEP* and control genes (*ATP8B1*) in family 2 (patient and his mother). (a) Gels and (b) graph of the optical density ratio between the *BSEP* and the *ATP8B1* PCR products. The patient/mother DNA concentration ratio was 0.94 for *ATP8B1* (control) and 0.52 for *BSEP*.

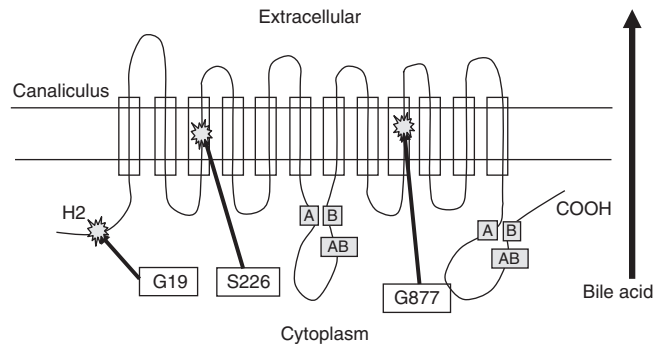


Figure 5 Schematic representation of the *BSEP* gene structure and the mutation locations. *BSEP* is a transmembrane protein that crosses the membrane 12 times. It has two domains of the ATP-binding cassette (ABC) transporters (green). The two mutations (S226L and G877R) are in the functional regions of the protein. One mutation (G19R) is in the N-terminal region. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

of many of our families may provide the first clue regarding the region of a new locus for PFIC. A better understanding of the genotype-phenotype correlation in PFIC will improve diagnoses and treatments of this disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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