Immunohistochemistry in Progressive Familial Intrahepatic Cholestasis (PFIC): Bridging Gap Between Morphology and Genetics

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Background/Aims: **A heterogeneous group of disorders caused by bile secretion and transport defects is progressive familial intrahepatic cholestasis (PFIC). PFIC has various subtypes with different presentations, laboratory findings, treatments, progression, and prognosis. Genetic analysis is the gold standard for diagnosis but is costly, time-consuming, and not readily available. In this study, immunohistochemistry (IHC) was evaluated as a tool for identifying subtypes of PFIC and differentiating them from other causes of pediatric cholestasis.** ***Methods:*** **The study included genetically confirmed PFIC (n = 40) and non-PFIC group (n = 20). Clinical history and laboratory investigations were recorded from the hospital information system. PFIC subtypes 1,2,3,4,5, and 6 showed the genetic mutation in ATP8B1, ABCB11, ABCB4, tight junction protein 2 (TJP2), NR1H4, and MYO5B, respectively. IHC has been applied for bile salt export pump (BSEP), multidrug resistance protein 3 (MDR3), TJP2, Claudin 1, farnesoid X receptor (FXR), and MYO5B.** ***Results:*** **IHC staining for BSEP, MDR3, TJP2, and MYO5B was positive in 100% of PFIC 1 and negative in 90.9%, 84.6%, 100%, and 100%, respectively, of the PFIC subtypes 2, 3, 4, and 6. Significant differences were noted between PFIC and non-PFIC patients for BSEP ($P = 0.044$), MDR3 ($P = 0.022$), and TJP2 ($P < 0.001$). In comparison with the non-PFIC patients, BSEP's sensitivity and specificity for diagnosing PFIC 2 was 90.9% and 95%, MDR3's for diagnosing PFIC 3 was 84.6% and 95%, TJP2 for PFIC 4 was 100% and 95%, and MYO5B''s for PFIC 6.** ***Conclusion:*** **Immunostaining for the markers BSEP, MDR3, TJP2, and MYO5B can differentiate various PFIC subtypes and distinguish between PFIC and non-PFIC patients. (J Clin Exp Hepatol 2025;15:102562)**

Neonatal cholestasis is approximately one per 2500 term infants.[1] The common causes are biliary atresia, genetic causes, and metabolic causes.[2] The term progressive familial intrahepatic cholestasis (PFIC) refers to an array of disorders that result from bile transport and secretion defects.[3] Genetically confirmed PFIC cases accounts for 12%–13% of cholestatic disorders among infants and children[4] and 10%–15% of children who need liver transplantation.[5] Mutations in the genes encoding these bile acid transporters and their regulators lead to bile salt accumulation in the hepatic parenchyma. These salts are toxic due to their detergent nature.[6]

It has been shown that three prominent subtypes of PFIC, namely PFIC1, PFIC2, and PFIC3, are caused by mutations in the ATP8B1 gene that encodes FIC1, the ABCB11 gene that encodes a bile salt export pump (BSEP) and the ABCB4 gene that encodes multidrug resistance protein 3 (MDR3).[7–9] PFIC 3 has a high Gamma-glutamyl transferase (GGT) cholestasis, while the others have normal or low GGT values. It has been found that two-thirds of subjects with normal or low GGT cholestasis do not carry any mutations at the ATP8B1 or ABCB11 genes.[5] A detailed mutational analysis of patients with this phenotype revealed three additional subtypes, including PFIC 4 due to loss of function of tight junction protein 2 (TJP2), which impairs Claudin-1 localization,[10,11] PFIC 5 occurs when NR1H4 mutations result in farnesoid X receptor (FXR) deficiency,[12,13] and PFIC 6 occurs when MYO5B mutations impair BSEP and MDR3 trafficking to canalicular surfaces.[14] A limited number of studies, mostly case reports or small case series, limits the true incidence of newer PFIC variants.

Keywords: cholestasis, progressive familial intrahepatic cholestasis, immunohistochemistry, gamma-glutamyl transferase, fibrosis
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Abbreviations: ABCB11: ATP binding cassette family B member 11; ABCB4: Adenosine triphosphate-binding cassette subfamily B member 4; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATP8B1: Adenosine triphosphate, type 8B, member 1; BSEP: Bile salt export pump; DB: Direct bilirubin; FXR: Farnesoid X receptor; FIC1: Intrahepatic cholestasis-1 protein; GGT: gamma-glutamyl transferase; IHC: Immunohistochemistry; MDR3: Multidrug resistance 3; MYO5B: Myosin 5B; NR1H4: Nuclear receptor subfamily 1, group H, member 4; PFIC: Progressive familial intrahepatic cholestasis; TB: Total serum bilirubin; TJP2: Tight junction protein 2

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All PFIC subtypes have different presentations, laboratory findings, treatments, disease progression, and prognoses. Genetic analysis is the gold standard for diagnosis; however, this approach has always been challenging given the costly nature of the technique and lack of availability, especially in resource-constrained countries such as India. Biopsy, histopathology, and immunohistochemical examination are simple, cost-effective diagnostic modalities for determining or narrowing the differential diagnosis.

There are few published works of literature on the role of immunohistochemistry (IHC) in PFIC, and the limited available IHC panels include BSEP and MDR3 only. In this study, we determined if an expanded immunohistochemistry panel could distinguish PFIC from other causes of pediatric cholestasis and identify different subtypes of PFIC.

MATERIALS AND METHODS

Study Population

A prospective study was conducted between January 2019 and December 2021 in the Pathology and Pediatric Gastroenterology Departments. The study was approved by the ethical committee of our institute (Ethical clearance id: 2020-5-IMP-EXP-14), and written informed consent was obtained. The PFIC case study group (n = 40) included all the children presented with phenotypic PFIC (neonatal or infantile cholestasis and pruritus) and has done genetic mutational analysis as a part of the management protocol. We excluded patients who had non-pathogenic mutational analysis for PFIC genes. Patients with other confirmed causes of neonatal cholestasis were taken as controls. The control group (non-PFIC; n = 20) had cases of biliary atresia, Alagille syndrome, bile acid synthetic defect, galactosemia, and Niemann–Pick disease diagnosed by positive per-operative cholangiogram, jagged-1 mutation, urine mass spectrometry/genetic analysis, positive Galactose-1-phosphate uridyltransferase (GALT) assay, and enzymatic analysis, respectively. PFIC group includes the subtypes 1 (n = 8), 2 (n = 11), 3 (n = 13), 4 (n = 6), 5 (n = 1), and 6 (n = 1), according to the genetic mutations seen in ATP8B1, ABCB11, ABCB4, TJP2, NR1H4, and MYO5B, respectively. The non-PFIC control group includes patients with biliary atresia (n = 8), bile acid synthesis defects (n = 6), Alagille syndrome (n = 3), galactosemia (n = 2), and Niemann–Pick disease (n = 1).

Clinical and Laboratory Workup

A detailed clinical evaluation and laboratory investigations were obtained from the Hospital Information System, including liver function tests, hemograms, coagulation profiles, viral markers, and genetic analysis. Diagnostic workup of the cases and controls was noted, including the genetic analysis, hepatobiliary iminodiacetic acid scan, urine non-glucose reducing sugar, GALT assay, enzymatic assay, and magnetic resonance cholangiopancreatography (MRCP).

Histopathology and Immunohistochemistry

The liver biopsies were fixed with 10% buffered formalin and then processed to form paraffin wax blocks. Following tissue sectioning, the slides were stained with hematoxylin and eosin (H&E), Masson's trichrome, and Gomori's reticulin to determine histology characteristics. The histological features were classified as absent (0) or present.[1] Metavir fibrosis scoring to stage fibrosis (F0: no fibrosis, F1: portal, F2: periportal, F3: bridging, and F4: cirrhosis) was done. F3 and F4 were considered significant stages for fibrosis. Paraffin wax blocks from case (PFIC) and control (non-PFIC) patients were sectioned at 4 μm and immunostained (Ventana Biosystems) with primary antibodies BSEP, MDR3, TJP2, CLDN1, FXR, and MYO5B in a phasic manner according to the algorithm described in Figure 1.

Various antibody clones used were described in the table below. Normal liver tissue (uninvolved liver tissue from gall bladder carcinoma patients) was included as a positive control for all the immunohistochemical staining.

Protein	Catalog No.	Clonality	Raised In	Company	Dilution
BSEP	HPA019035	Polyclonal	Rabbit	Sigma–Aldrich	1:2000
MDR3	HPA053288	Polyclonal	Rabbit	Sigma–Aldrich	1:100
TJP2	HPA001813	Polyclonal	Rabbit	Sigma–Aldrich	1:100
Claudin 1	2H10D10	Monoclonal	Rabbit	Invitrogen	1:200
FXR	AV33672	Polyclonal	Rabbit	Sigma–Aldrich	1:50
MYO5B	PA5-59344	Polyclonal	Mouse	Invitrogen	1:100

Positive immunostaining for BSEP, MDR3, and TJP2 was observed on the canalicular surface. CLDN1 stained the tight junctions of cholangiocytes. MYO5B had a membranous and weakly granular cytoplasm, and FXR was expressed in the nuclei of the hepatocytes. Sections were evaluated by a trained hepatopathologist (NN, NK), and the findings were reviewed by a dedicated

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hepatopathologist (CB) who was unaware of the diagnosis. Immunohistochemistry was repeated in patients whose morphology and genetic profile were discrepant. The immunohistochemical staining was interpreted in a semi-quantitative manner as described below.

IHC Score	Intensity	Percentage of the positive cells	Interpretation
0	Absent	Absent	Negative
1	Focal or weak	<10%	Positive
2	Moderate	10%–50% (reduced)	
3	Strong	>50%	

Statistical Analysis

A statistical analysis was conducted using SPSS software (SPSS, Chicago, IL). Quantitative variables are represented by mean ± standard deviation, and categorical variables are represented by frequencies (%). Categorical variables were compared between groups using the appropriate Chi-square or Fisher exact test. On applying the Fisher exact test, the proportion of the patients with different histological features was compared among the various subtypes of PFIC. A P value of <0.05 indicated statistical significance.

RESULTS

Study Population Characteristics

There were 60 children in this study, 40 of whom were in the PFIC group and 20 in the non-PFIC group. In the PFIC group, the median age is 9[5–15] months, while in the non-PFIC group, it is 6.5 (4.1–10.3) months. Male to female sex distribution in percentage between the groups are 65/35 and 75/25, respectively. Median age and sex were not significantly different between the two groups (P > 0.05 for both; P = 0.224, P = 0.432 respectively).

Clinical and Laboratory Characteristics of the PFIC and Non-PFIC Groups

The PFIC group had significantly more pruritus (83.9% vs. 16.1%; P = 0.002), and the non-PFIC group had significantly more clay-colored stools (50% vs. 10%; P = 0.004). The PFIC and non-PFIC groups did not differ significantly in hematological parameters (hemoglobin, total leukocyte count, and platelet count), transaminase levels, alkaline phosphatase levels, gamma-glutamyl transferase (GGT) levels, or international normalized ratio. GGT levels were raised in PFIC 3 (10/13; 76.9%) of the PFIC group (Table 1).

Histological Characteristics of the Study Population (PFIC Patients) (Table 2; Figure 2)

PFIC subgroups may have overlapping histological features. There was a significant difference in the proportions of duct proliferation, canalicular cholestasis, pseudo rosetting of hepatocytes, thickened hepatic arterioles, and fibrosis (P < 0.05). The rest of the histological features were statistically equal among the groups (P > 0.05). Pseudo rosette formation (7; 87.5%) and canalicular cholestasis (7; 87.5%) are seen in PFIC1. Liver biopsies of the PFIC3 cohort revealed moderate to marked ductular proliferation (9; 69.2%). Significant fibrosis to cirrhosis was

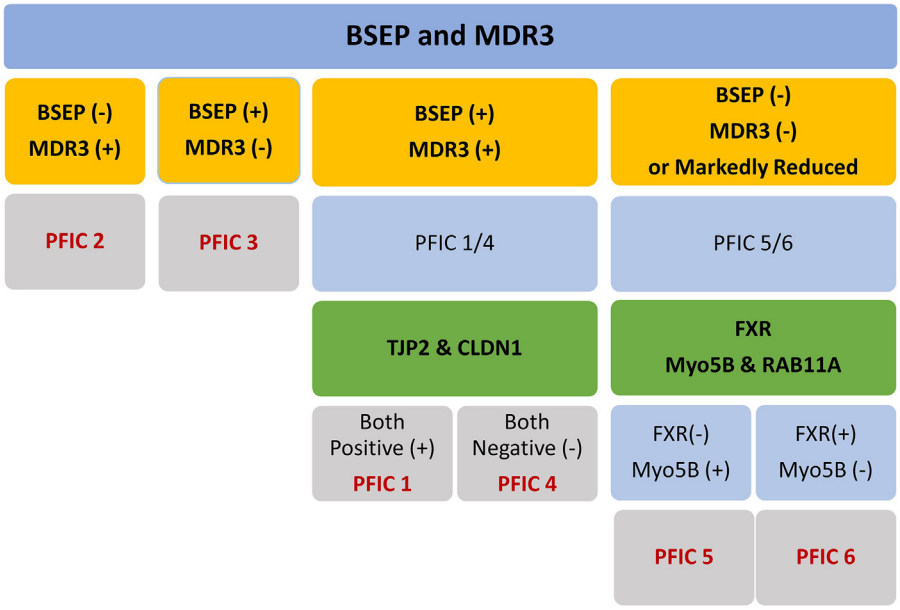

Figure 1 Algorithmic approach for immunohistochemistry.

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Table 1 Distribution of Demographic and Clinical Variables Between the PFIC (Patients) and Non-PFIC (Controls) Groups (n = 60).

Clinical & laboratory parameters	PFIC 1 (n = 8)	PFIC 2 (n = 11)	PFIC 3 (n = 13)	PFIC 4 (n = 6)	PFIC 5 (n = 1)	PFIC 6 (n = 1)	Non-PFIC (n = 20)	*P* value
Age (months)	10 ± 9.11	8.07 ± 5.36	50.41 ± 54.11	7.58 ± 3.77	2	36	9.2 ± 10.4	0.068
Male	5 (62.5%)	7 (63.64%)	10 (76.92%)	2 (33.33%)	1 (100%)	1 (100%)	15 (75%)	0.432
Hemoglobin	9.23 ± 1.14	10.91 ± 2.1	9.59 ± 2.6	9.28 ± 1.9	15.5	10.9	11.1 ± 2.2	0.085
TLC	16.0 ± 5.7	17.34 ± 6.6	11.19 ± 5.7	18.95 ± 4.1	7	13	15.8 ± 9.8	0.699
Platelet	497.3 ± 242.5	356.3 ± 111.8	229.9 ± 139.7	305.7 ± 114.6	153	557	346.4 ± 175.4	0.830
INR	1.06 ± 0.1	1.71 ± 1.2	1.39 ± 0.8	1.33 ± 0.5	1.29	0.91	1.43 ± 0.8	0.861
T. BIL.	14.11 ± 5.8	10.45 ± 8.9	6.3 ± 6.9	13.58 ± 6.15	15.6	0.3	8.88 ± 6.8	0.524
D. BIL.	9.94 ± 4.6	6.54 ± 5.3	3.63 ± 3.9	9.17 ± 4.1	1.2	0.1	5.81 ± 5	0.686
SGOT	254.3 ± 275	337.5 ± 187.9	263.15 ± 329.8	346.33 ± 417.1	112	64	436.1 ± 467.3	0.128
SGPT	181.3 ± 232	191.09 ± 139.1	170.54 ± 121.5	101 ± 88	45	47	209.5 ± 153.5	0.248
ALK PHOS	489.7 ± 162.1	526 ± 271.5	510.1 ± 325.5	298.3 ± 136.2	754	301	530.1 ± 460.1	0.588
GGT	44.6 ± 27.5	34.4 ± 16.1	209.1 ± 126.7	40.8 ± 11.1	50	19	177.2 ± 231.6	0.062

Comparisons were made between cases and controls. Data are presented in Number (%), compared by Chi-square test. Mean ± SD, compared by independent samples *t* test. ***P* < 0.05 significant**.
GGT, gamma-glutamyl transferase; PFIC, progressive familial intrahepatic cholestasis.

Table 2 Distribution of the Histological Features Among the Various Subtypes of PFIC (n = 39).

Histopathological features		PFIC 1 (n = 8)	PFIC 2 (n = 11)	PFIC 3 (n = 13)	PFIC 4 (n = 6)	PFIC 6 (n = 1)	*P* value
Duct proliferation	0	7 (87.5)	9 (81.8)	4 (30.8)	4 (66.7)	1 (100)	**0.019**
	1	1 (12.5)	2 (18.2)	9 (69.2)	2 (33.3)	0 (0)	
Duct paucity	0	5 (62.5)	10 (90.9)	10 (76.9)	5 (83.3)	1 (100)	0.643
	1	3 (37.5)	1 (9.1)	3 (23.1)	1 (16.7)	0 (0)	
Portal inflammation	0	6 (75)	8 (72.7)	6 (46.2)	3 (50)	1 (100)	0.085
	1	2 (25)	3 (27.3)	7 (53.8)	3 (50)	0 (0)	
Giant cells	0	8 (100)	4 (36.4)	11 (84.6)	5 (83.3)	1 (100)	0.716
	1	0 (0)	7 (63.6)	2 (15.4)	1 (16.7)	0 (0)	
Canalicular cholestasis	0	1 (12.5)	5 (45.5)	11 (84.62)	6 (100)	1 (100)	**0.002**
	1	7 (87.5)	6 (54.5)	2 (15.4)	0 (0)	0 (0)	
Ballooning degeneration	0	8 (100)	4 (36.4)	12 (92.3)	4 (66.7)	1 (100)	0.148
	1	0 (0)	7 (63.6)	1 (7.69)	2 (33.33)	0 (0)	
Lobular inflammation	0	7 (87.5)	3 (27.27)	12 (92.3)	3 (50)	1 (100)	0.613
	1	1 (12.5)	8 (72.7)	1 (7.69)	3 (50)	0 (0)	
Fibrosis	1,2	6 (75)	7 (63.6)	3 (23.1)	1 (16.7)	1 (100)	**0.025**
	3,4	2 (25)	4 (36.4)	10 (76.9)	5 (83.3)	0 (0)	
Central vein sclerosis	0	7 (87.5)	9 (81.82)	12 (92.31)	6 (100)	0 (0)	0.228
	1	1 (12.5)	2 (18.18)	1 (7.69)	0 (0)	1 (100)	
Pseudorosettes	0	1 (12.5)	5 (45.5)	9 (69.2)	3 (50)	1 (100)	**0.012**
	1	7 (87.5)	6 (54.5)	4 (30.8)	3 (50)	0 (0)	
Thickened artery	0	8 (100)	10 (90.91)	13 (100)	3 (50)	1 (100)	**0.016**
	1	0 (0)	1 (9.09)	0 (0)	3 (50)	0 (0)	

Data are presented in number (%) compared by the Fisher exact test. ***P* value <0.05 significant**.
PFIC, progressive familial intrahepatic cholestasis.

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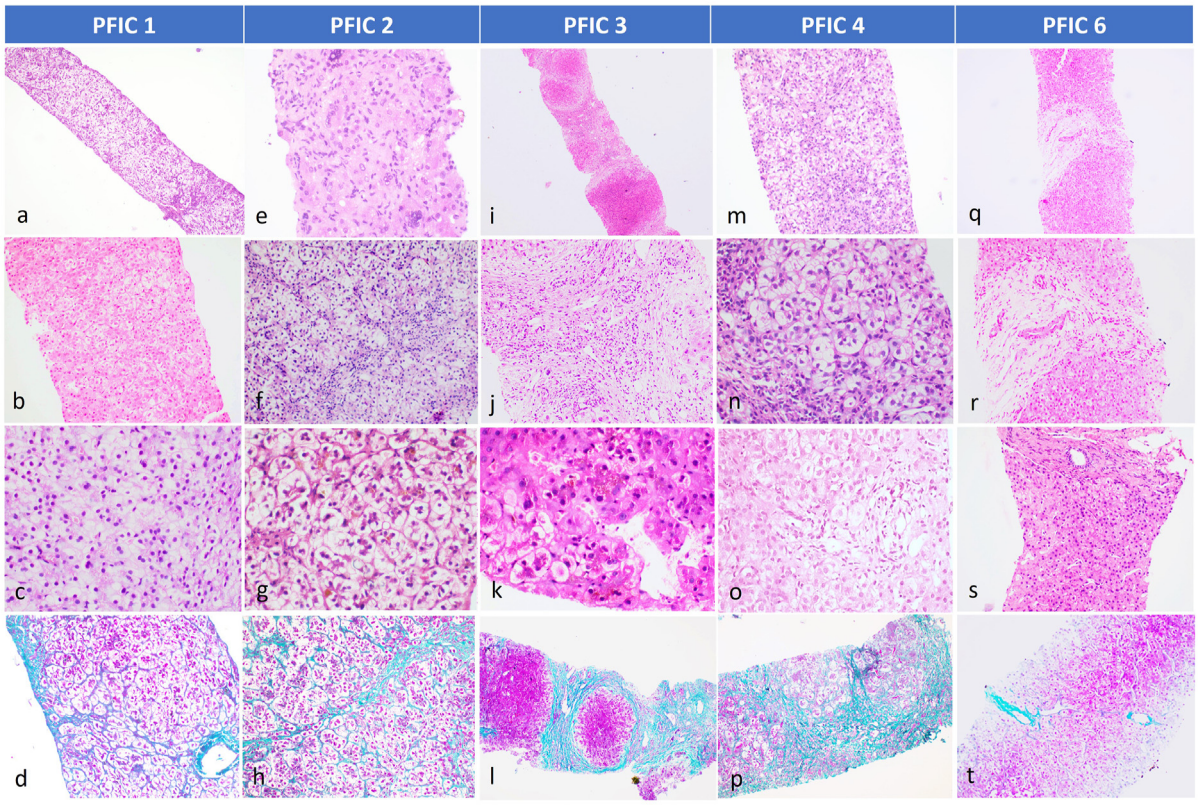

Figure 2 PFIC 1: Biopsy showing acinar disarray, bland cholestasis (a; 100x, b; 200x; H&E), and extensive pseudo rosette formation (c; 400x; H&E). Fibrosis is mild, limited to portal and periportal area (d; 200x; MT). PFIC 2: Liver histology revealed giant cell transformation and ballooning degeneration (e; 200x; H&E), severe lobular inflammation (f; 400x; H&E), canalicular cholestasis (g; 400 x; H&E), and 8; 72.7%), and mild portal, periportal, perisinusoidal fibrosis (h; 200x; MT). PFIC 3: Biopsy showing distorted architecture and nodule formation (l; 100x; H&E), portal inflammation and moderate ductular proliferation (j; 200x; H&E), ballooning degeneration and canalicular cholestasis (k; 400x; H&E). Significant fibrosis is noted with the formation of micronodules (l; 100x; MT). PFIC 4: There is portal inflammation and lobular inflammation (m; 200x; H&E), pseudo rossetting of hepatocytes (n; 400x; H&E), arterial thickening (o; 400x; H&E), and advanced fibrosis (p; 100x; MT). PFIC 6: A liver biopsy revealed near-normal histology with no lobular or portal inflammation (q; 100x; H&E). Portal tracts are expanded by thick fibrosis (r; 200x; H&E). Focal canalicular cholestasis 200x; H&E) and central vein sclerosis (t; 200x; MT) is noted. H&E, hematoxylin and eosin; MT, Masson's trichrome; PFIC, progressive familial intrahepatic cholestasis.

noted in PFIC3 (10; 76.9%) and PFIC4 (5; 83.3%). Arterial thickening was reported in 4 (4/40; 10%) of the PFIC patients, most of which were PFIC4 (3; 50%). PFIC 6 showed near-normal liver histology with central vein sclerosis.

Immunostaining for BSEP, MDR3, TJP2, FXR, and MYO5B in the Studied Groups (PFIC and Non-PFIC)

Immunohistochemistry Within PFIC Subtypes

Immunohistochemistry (IHC) was applied in 39 cases, as liver biopsy was unavailable for the case, revealing the FXR mutation. Immunohistochemistry in PFIC 1 patients (n = 8) revealed positive canalicular staining for BSEP, MDR3, and TJP2. Claudin 1, FXR, and MYO5B exhibited positive immunoreactivity at their respective locations. TJP2 and FXR expression was reduced (score 2) in two patients; however, these patients were positive. PFIC 2 patients (n = 11) showed complete loss of BSEP expression in ten patients (10; 90.9%) and moderate reduction (∼40%; score 2) in one patient (1; 9.1%) on immunohistochemistry. MDR3 (10; 90.9%), TJP2 (10; 90.9%), claudin 1 (10; 90.9%), FXR (11; 100%), and MYO5B (11; 100%) were positive. In PFIC 3 subgroup (n = 13), the immunohistochemistry revealed complete loss (negative) of MDR3 expression in eleven patients (11; 84.6%) and moderate reduction (∼30%, positive) in two patients (2; 15.4%). BSEP (13; 100%), TJP2 (11; 84.6%), claudin 1 (12; 92.3%), FXR (13; 100%), and MYO5B (13; 100%) were positive. PFIC 4 patients (n = 6), the immunohistochemistry showed a complete or marked decrease in TJP2 and claudin expression in all the patients (6; 100%). BSEP (6; 100%), MDR3 (6; 100%), FXR (6; 100%), and MYO5B (6; 100%) were positive. PFIC 6 patient (n = 1) had an abnormal granular cytoplasmic staining for MYO5B (1; 100%) and an abnormal pattern for BSEP (absent) and MDR3 (thick subcanalicular staining). The remaining immunomarkers, TJP2, claudin1, and FXR, showed intact staining.

IHC staining for BSEP, MDR3, TJP2, and MYO5B was negative in 90.9%, 84.6%, 100%, and 100%, respectively, of

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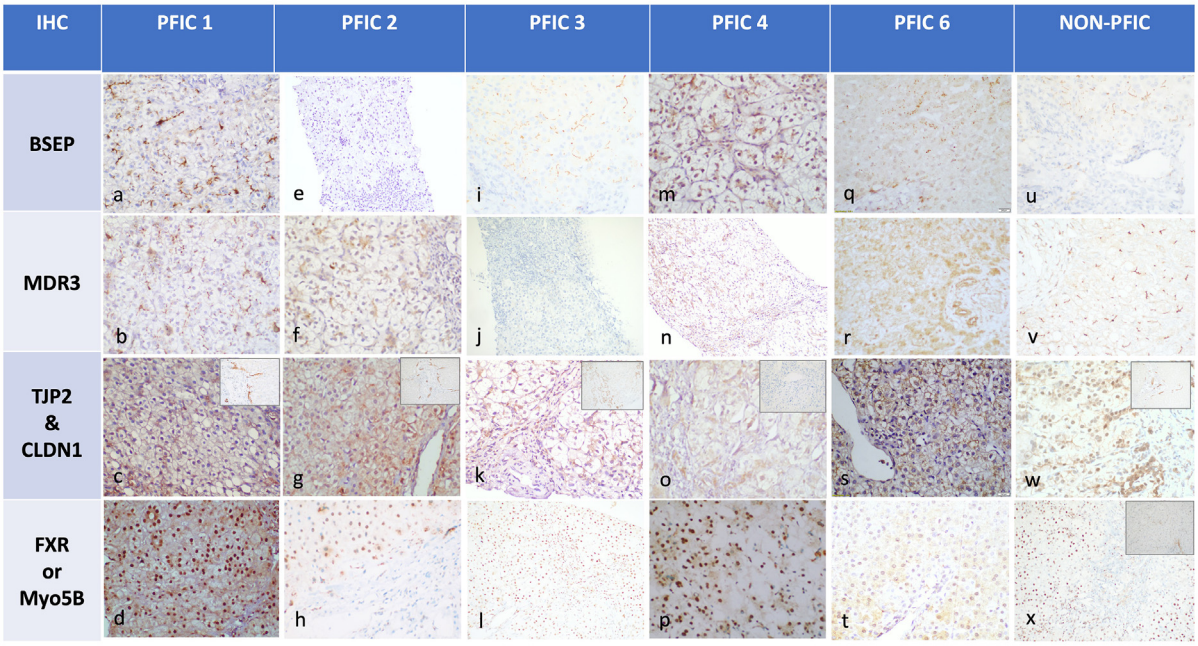

Figure 3 PFIC 1: Immunohistochemistry shows positive canalicular staining for BSEP (a; 400x; IHC), MDR3 (b; 400x; IHC), TJP2 (c; 400x; IHC), claudin 1 in cholangiocytes (inset), and nuclear staining for FXR (d; 400x; IHC). PFIC 2: Negative staining for BSEP (e; 200x; IHC), and positive staining for MDR3 (f; 400x; IHC), TJP2 (g; 400x; IHC, claudin 1 in inset), and FXR (h; 400x; IHC). PFIC 3: BSEP is positive (i; 400x; IHC) and MDR3 is negative (j; 200x; IHC). TJP2 (k; 400x; IHC, claudin1 in inset), and FXR (l; 400; IHC) are positive. PFIC 4: Immunostaining for BSEP (m; 400x; IHC), MDR3 (n; 200x; IHC), and FXR (p; 400x; IHC) are positive, whereas TJP2 and claudin 1 are negative (o; 400x; IHC, claudin 1 in inset). PFIC 6: BSEP (q; 400x; IHC) and MDR3 (r; 400x; IHC) show abnormal subcanalicular staining, TJP2 shows positive staining (s; 400x; IHC), and MYO5B shows moderate granular cytoplasmic positivity (t; 400x; IHC). In non-PFIC patients BSEP (u; 400x; IHC), MDR3 (v; 400x; IHC), TJP2 (w; 400x; IHC, claudin 1 in inset), FXR (x; 400x; IHC) and MYO5B in inset. FXR, farnesoid X receptor; IHC, immunohistochemistry; MDR3, multidrug resistance protein 3; PFIC, progressive familial intrahepatic cholestasis; TJP2, tight junction protein 2.

the PFIC subtypes 2, 3, 4, and 6. All the IHC markers were positive for PFIC1 (8; 100%) (Figure 3).

Immunohistochemistry in Non-PFIC Patients

Negative immunohistochemistry for BSEP, MDR3, and claudin1 was not restricted to PFIC patients. One patient (5%) of the non-PFIC group, diagnosed with biliary atresia, showed negative BSEP immunostaining. One patient (5%) of galactosemia had negative immunostaining for MDR. Two patients (10%), one with biliary atresia and bile acid synthetic defect, showed negative claudin-1 immunostaining. TJP2, FXR, and MYO5B immunostaining are preserved in non-PFIC (control) patients.

Immunohistochemistry in PFIC Versus Non-PFIC Patients

Immunohistochemistry revealed significant differences between PFIC and non-PFIC patients for BSEP ($P = 0.044$), MDR3 ($P = 0.022$), and TJP2 ($P < 0.001$). It was not found significant for claudin 1 ($P = 0.308$), FXR ($P = 0.544$), and MYO5B ($P = 0.99$) (Table 3). No patient in the PFIC 5 (FXR) subgroup and only one patient in the PFIC 6 (MYO5B) subgroup limit the calculation of the statistical significance.

Sensitivity and Specificity of the Immunohistochemical Markers

Compared with the non-PFIC patients, the sensitivity and specificity of the immunomarker BSEP for diagnosing the PFIC 2 patient was 90.9% (58.7%–99.8%) and 95% (73.1%–99.9%), respectively, of MDR3 for diagnosing PFIC 3 patients was 84.6% (54.6%–98.1%) and 95% (73.1%–99.9%), respectively, of TJP2 for PFIC 4 patients was 100% (54.1%–100%) and 95% (73.1%–99.9%), respectively, and MYO5B for PFIC 6 patients, it was 100% (2.5%–100%) and 100% (83.2%–100%), respectively (Table 4).

DISCUSSION

Clinical, Laboratory, and Histological Profiles of PFIC Subtypes

PFIC 1 shows canalicular cholestasis and extensive pseudo rosetting. Clayton et al. describe preserved lobular architecture with canalicular cholestasis, extensive hepatocytic pseudo rosettes, and minimal portal fibrosis in liver biopsies of PFIC 1 patients.[3,8] Histologically, early PFIC2 exhibits neonatal hepatitis-like changes, including giant cell transformation, inflammation, and canalicular

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Table 3 Distribution of Immunohistochemical Findings Among Various Subtypes of PFIC (Patients; n = 39) and Non-PFIC (Control; n = 20) Patients.

S. No	IHC Ab	Negative (0,1) Positive (2,3)	PFIC 1 (n = 8)	PFIC 2 (n = 11)	PFIC 3 (n = 13)	PFIC 4 (n = 6)	PFIC 6 (n = 1)	Non-PFIC (n = 20)	*P* value
1	**BSEP**	Negative	0	10	0	0	1	1	**0.0442**
		Positive	8	1 (1; reduced)	13	5 (1; reduced)	0	19 (3:reduced)	
2	**MDR3**	Negative	0	1	11	0	1	1	**0.0219**
		Positive	8	10	2 (2; reduced)	6 (1; reduced)	0	19 (4; reduced)	
3	**TJP2**	Negative	0	1	2	6	0	0	**0.0218**
		Positive	8 (2; reduced)	10	11	0	1	20 (9; reduced)	
4	**CLDN1**	Negative	0	1	1	6	0	2	0.308
		Positive	8 (1; reduced)	10	12 (1; reduced)	0	1	18 (3; reduced)	
5	**FXR**	Negative	0	0	0	0	0	0	0.544
		Positive	8 (2; reduced)	11	13	6	1	20 (2; reduced)	
6	**MYO5B**	Negative	0	0	0	0	1	0	0.99
		Positive	8	11	13	6	0	20 (8; reduced)	

Data are presented in Number (%) compared by the Fisher exact test. ***P* value <0.05 significant**.
Fisher exact test was used to test the association of cases and controls with the severity of the histopathological features of the various markers. The result showed that other markers were significantly associated with cases and controls except for the severity of the FXR and RAB11A.
BSEP, bile salt export pump; FXR, farnesoid X receptor; IHC, immunohistochemistry; MDR3, multidrug resistance protein 3; PFIC, progressive familial intrahepatic cholestasis; TJP2, tight junction protein 2.

Table 4 Sensitivity and Specificity of the Immunohistochemical Markers Within PFIC Subtypes Compared With the Non-PFIC (Control; n = 20) Patients.

Immunohistochemistry		PFIC patients		Non-PFIC patients		Sensitivity	Specificity
		Positive	Negative	Positive	Negative		
BSEP	PFIC 2 (n = 11)	1	10	19	1	90.9% (58.7%–99.8%)	95% (73.1%–99.9%)
MDR3	PFIC 3 (n = 13)	2	11	19	1	84.6% (54.6%–98.1%)	95% (73.1%–99.9%)
TJP2	PFIC 4 (n = 6)	0	6	19	1	100% (54.1%–100%)	95% (73.1%–99.9%)
Myo5b	PFIC 6 (n = 1)	0	1	20	0	100% (2.5%–100%)	100% (83.2%–100%)

BSEP, bile salt export pump; MDR3, multidrug resistance protein 3; PFIC, progressive familial intrahepatic cholestasis; TJP2, tight junction protein 2.

cholestasis.[15] PFIC 3 presents in older children, the only subtype associated with high GGT levels. According to Dröge *et al.* (2017), in a cohort of 427 patients with suspected genetic cholestasis, PFIC-3 patients had high GGT and alkaline phosphatase levels. They exhibited a later onset of symptoms than PFIC-1 or PFIC-2 patients.[16,17]

In PFIC 3, the liver histology is characterized by ductular proliferation.[3] PFIC 4 exhibits tight junction disruption, leading to severe cholestatic liver disease, intracellular cholestasis, and giant cell transformation.[10] Another feature observed was the thickened hepatic arteries, which need to be explored in further studies. The PFIC-6 patients show near-normal liver histology with minimal portal fibrosis. Significant fibrosis is seen in PFIC 3 and PFIC 4.

The laboratory and histopathological features are overlapping. Our understanding of the disease spectrum in Indian children is limited by the lack of advanced diagnostic facilities (genetic analysis). Immunostaining might enhance our understanding of this disease by distinguishing PFIC from other causes of pediatric cholestasis and identifying subtypes of PFIC.

Role of Immunohistochemistry in PFIC

Among the normal or low GGT PFIC, PFIC1 and PFIC2 may have overlapping clinical, biochemical, and histological presentation. Immunohistochemistry helps differentiate the two. A retrospective review of 62 children with normal GGT PFIC found genetic mutations in ATP8B1 and ABCB11. PFIC1 mutations were found in 13 patients, PFIC2 mutations in 39 patients, and the origin was unknown for 10 patients. PFIC2 was suspected of a high serum alanine aminotransferase level, severe lobular lesions accompanied by giant hepatocytes, early liver failure, and negative BSEP canalicular staining. There is a loss of immunohistochemically demonstrable BSEP expression associated with mutations in ABCB11.[15,18] PFIC 2 patients

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with negative BSEP expression constituted 90.9% in our study. In addition, Davit-Spraul *et al.* and Kimberley Evason *et al.* reported negative BSEP expression in 91.7% and 83.3% of PFIC2 patients with homozygous or heterozygous ABCB11 mutations, respectively.[18–20] According to them, a minority of patients with clinically detected PFIC2 and ABCB11 mutations may still have immunohistochemically detectable BSEP, though the protein may not be fully functional or quantitatively sufficient.[20] One patient exhibited moderate to markedly reduced (20%–30% expression; score 2) BSEP expression in the canaliculi. A positive expression of BSEP can be explained by the fact that some ABCB11 mutations with disease-causing effects result in abnormal protein locations or functions while retaining antigenicity despite homozygous or heterozygous ABCB11 mutations.[21] Reduced FXR expression (score 2) is noted in two cases of PFIC1 deficiency (25%), which represents a consequence of a cholestatic phenotype.[22]

A significant difference ($P = 0.0219$) was found between the PFIC and non-PFIC groups for MDR3-negativity (32.5% versus 5%). Immunostaining for MDR3 was negative for PFIC3 (84.6%), PFIC2 (7.7%), and PFIC6 (7.7%). Moreover, two patients, one with missense mutations, showed reduced or normal MDR3 canalicular expression. In a study by Colombo *et al.*, 71.42% of PFIC3 patients showed no or faint MDR3 staining. Of these, 80% had homozygous ABCB4 mutations, while the remaining 20% had homozygous mutations.[23] A similar report by Wendeum *et al.* showed that, in PFIC3 patients with ABCB4 gene mutations, MDR3 immunostaining revealed a complete absence of canalicular staining but faint or sometimes normal canalicular staining.[24]

PFIC patients had higher rates of negative TJP2 expression and abnormal claudin-1 immunostaining (23%, 20.5%) than non-PFIC patients (0%, 10%) (P 0.022; 0.308). Negative TJP2 immunostaining was noted for PFIC4 (100%), PFIC2 (9.1%), and PFIC3 (15.4%). Sambrotta et al. studied 33 children with chronic cholestatic liver disease, low GGT, and known to harbor a homozygous mutation in TJP2. TJP2 was not detected by immunohistochemistry or western blotting with an antibody against its C-terminal epitope. Claudins are integral to tight junction structures.[25,26] The immunohistochemistry analysis of CLDN1 expression showed tight junction markers in controls. A significantly reduced staining was observed in liver tissue of patients lacking TJP2, indicating that CLDN1 cannot localize without TJP2.[10]

A mutation in NR1H4 on chromosome 12q23 causes PFIC5. The FXR nuclear hormone receptor regulates bile acid homeostasis and metabolism, and ABCB11 and ABCB4 are the direct targets. In their study, Gomez Ospina *et al.* reported four cases of neonatal cholestasis associated with mutations in NR1H4, which encodes the farnesoid X receptor (FXR). All the patients had neonatal onset with rapid progression to end-stage liver disease, vitamin K-independent coagulopathy, low-to-normal serum gamma-glutamyl transferase activity, elevated serum alpha-fetoprotein, and undetectable liver bile salt export pump (ABCB11) expression. Immunohistochemical analysis of BSEP, MDR3, and FXR in the liver revealed no immunostaining for BSEP or FXR in any of the four patients.[12] FXR is retained in other PFIC subtypes.

A molecular motor associated with actin (MYO5B) interacts with a recycling endosome-associated RAB family protein (particularly the RAS-related protein RAB11A), which is necessary for the proper functioning of polarized epithelial cells, including positioning recycling endosomes subcellularly and forming the bile canaliculus. BSEP, MYO5B, and MDR3 immunostaining were abnormal in one patient with MYO5B mutation. Due to an alteration of the MYO5B/RAB11A interaction, the expression of BSEP and MDR3 is decreased, thereby worsening cholestasis. In five patients with a familial intrahepatic cholestasis-like phenotype and normal serum gamma-glutamyl transferase activity, Gonzale *et al.* identified MYO5B mutations.[14] Two patients have been immunostained for BSEP, MDR3, and MYO5B. A granular and patchy pattern was observed in the subcanalicular area of patients with BSEP and MDR3 staining. Two patients had abnormal MYO5B immunoreactivity, characterized by intense and granular staining in the cytoplasm but not at the plasma membrane.[14] In a cohort of Chinese children with low GGT cholestasis, Qui *et al.* reported a prevalence of MYO5B deficiency in 20% of the previously undiagnosed patients. Liver biopsy specimens from five MYO5B-mutated patients demonstrated coarse granular dislocation of MYO5B at canaliculi and abnormal distribution of the bile salt export pump (BSEP).[27]

Immunohistochemistry in Non-PFIC Patients

Negative immunohistochemistry for BSEP (5%), MDR3 (5%), and Claudin1 (10%) were also observed in the non-PFIC patients. Patients included are those with biliary atresia, galactosemia, and bile acid synthetic defects. Chen *et al.* described the adaptive downregulation of canalicular and sinusoidal uptake transporters in early obstructive cholestasis (BA). This could explain the absence of the BSEP and MDR3 proteins in liver biopsies of such patients.[28] The heterozygosity of ABCB4 has also been associated with an increase in hepatic CD8+ T-cells and natural killer lymphocyte responses to viral infection and ductal obstruction.[28] In patients with neonatal cholestasis with etiologies other than PFIC, the PFIC gene mutations were described by Liu *et al.* This requires genetic profiling of non-PFIC patients, such as those with galactosemia.[29] Those with severely cholestatic liver profiles show negative

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or significantly decreased claudin-1 immunostaining. A crucial role is played by tight junctions within the liver in cholestasis-induced increases in biliary permeability. The junctional proteins were studied after bile duct ligation and release of ligation, which is a severe cholestatic condition, by Maly *et al.* As a result of ligation, TJP-2, TJP-1, and occludin were upregulated but not claudins. A colocalization analysis showed a decrease in claudin proteins.[30]

This study demonstrated that immunostaining for BSEP, MDR3, TJP2, and MYO5B can identify PFIC subtypes. BSEP, MDR3, and TJP2 staining can be used to distinguish PFIC patients from non-PFIC patients.

Its limitation lies in the lack of immunohistochemical data in PFIC 5 and the small number of patients in PFIC 6. Nevertheless, our results suggest the possibility of conducting further studies of a larger PFIC cohort.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

(I) Dr Neha Nigam contributed to conception and design.
(II) Dr Neha Nigam, Dr Moinak Sen Sharma, and Dr Anshu Srivastava contributed to provision of study materials or patients.
(III) Dr Neha Nigam and Dr Moinak Sen Sharma contributed to collection and assembly of data.
(IV) Dr Neha Nigam, Dr Chhagan Bihari, and Dr Narendra Krishnani Prabhakar Mishra contributed to data analysis and interpretation.
(V) Dr Neha Nigam contributed to manuscript writing.
(VII) All authors contributed for final approval of manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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No data are presented or discussed.

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