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Next-generation sequencing panel test results in pediatric patients with progressive familial intrahepatic cholestasis: a single-center experience

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ABSTRACT

Objectives: The aim of this study is to reveal the diagnostic yield of the progressive familial intrahepatic cholestasis (PFIC) gene panel that we have used in the diagnosis of this patient group, which accounts for approximately 10% of cholestatic liver disease, and to report the clinical findings of our patients with the detected variants.

Methods: In this study, we retrospectively evaluated the results of molecular genetic analysis of pediatric patients whose PFIC gene panel contained the *ATP8B1, ABCB11, and ABCB4* genes.

Results: In 10 patients, 12 different variants were detected that could explain the PFIC clinical picture. Three of these variants were considered novel variants.

Conclusions: Our study demonstrates the usefulness of the NGS panel in diagnosing pediatric patients with PFIC findings. This diagnostic method also contributed to the variant spectrum of PFIC-related genes.

Keywords: Progressive familial intrahepatic cholestasis, novel, *ABCB11, ABCB4, ATP8B1*

Cholestasis, which may be intrahepatic or extrahepatic and caused by genetic or nongenetic multifactorial conditions, is the reduction or disruption of bile flow produced by hepatocytes and cholangiocytes [1]. insufficient bile flow due to hereditary or acquired diseases causes bile contents such as bilirubin, bile acids, and lipids to accumulate in the liver, resulting in high bilirubin and bile salt levels in the liver and blood and irregular lipid metabolism. While jaundice, itching, and clayey stools that develop due to hyperbilirubinemia are usually observed in patients, bleeding episodes in the form of intracranial hemorrhage is rare [2]. Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of genetic diseases that show signs of cholestasis and lead to liver failure. It is a rare disease with an estimated prevalence of 1-2 per 100,000 births, although the exact prevalence is unknown. In this group of diseases, which is inherited in an autosomal recessive manner, many different types have been defined, with the first three types being the best known [3]. PFIC1 and PFIC2 usually occur in infancy or early childhood. PFIC3 can occur later in infancy, childhood and even young adulthood. All three types are associated with hepatocellular transport system genes involved in bile formation. PFIC1 is caused by variants in the *ATP8B1* gene, re-

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sulting in the deficiency of familial intrahepatic cholestasis 1 (FIC1) protein. *ATP8B1* protein is located on the canalicular membrane of hepatocytes. It is responsible for transporting phospholipids (phosphatidylserinne and phosphaticylethanolamine) inside the canalicular membrane. PFIC2 is caused by the variants in the *ABCB11* gene, resulting in the deficiency of bile salt export pump (BSEP). 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 canaliculi against a concentration gradient. PFIC3 is caused by variants in the *ABCB4* gene, resulting in the deficiency of the multidrug resistant 3 (MDR3) protein. It is expressed in the canalicular membrane of hepatocytes and acts as a floppase responsible for the biliary secretion of phospholipids [4]. In recent years, the use of next-generation sequencing (NGS) has increased to obtain a molecular diagnosis in PFIC. NGS is a technology proposed for the molecular diagnosis of PFIC and compared to classic Sanger, NGS allows rapid sequencing with more information at lower costs [5].

In this study, the results of molecular genetic analysis of pediatric patients examined with cholestasis whose *ATP8B1, ABCB11*, and *ABCB4* genes responsible for PFIC types 1, 2, and 3 were sequenced by NGS in our laboratory were retrospectively evaluated.

METHODS

Thirty-seven patients who presented to the Medical Genetics Department Bursa Yüksek Ihtisas Training and Research Hospital with a preliminary diagnosis of PFIC between 2015 and 2019 were retrospectively evaluated. Informed consent for genetic testing was obtained from all patients or their legal guardians. The local clinical research ethics committee granted approval for the study (2011-KAEK-25, 2019/08-01).

An NGS platform (NextSeq 500, Illumina, San Diego, California, USA) was used for this study. The PFIC panel (An NGS platform (NextSeq 500, Illumina, San Diego, California, USA) was used for this study. The PFIC panel (PFIC Solution, Sophia™, Saint Sulpice, Switzerland) was studied in patients, and all steps were performed according to the original manufacturer's protocol. The raw data obtained were filtered and analyzed using the appropriate program (Sophia DDM, Saint Sulpice, Switzerland). Considering the clinical findings, and family history of the patients, variants that could be significant were determined. These significant variants, which were detected during the analysis of the PFIC panel and could be associated with any disease, were evaluated using the Human Gene Mutation Database (HGMD) [6]. This allowed us to determine whether the change had been reported in the literature and, if so, to which disease it was associated. For alterations not reported in the literature, classification by American College of Medical Genetics and Genomics (ACMG) criteria and frequency in population studies (gnomAD; Genome Aggregation Database) were determined using the Varsome Analysis Program (https://varsome.com/) [7, 8]. This panel includes PFIC types 1, 2, and 3 genes.

RESULTS

Patients who were referred with a prediagnosis of PFIC, particularly with evidence of cholestasis, were included in the study. In 10 (27%) of 37 patients whose PFIC gene panels were examined, variants were detected in the *ATP8B1, ABCB11,* and *ABCB4* genes that could be associated with the clinical picture of patients (Table 1). Nine of the patients had symptoms of cholestasis. One patient had no other findings except mild jaundice. Two patients, one with PFIC1 and other with PFIC2, underwent liver transplant. The mean age at diagnosis of patients with variants in the *ATP8B1, ABCB11* and *ABCB4* genes was 24 months. Consanguinity between parents was observed as 60%. A total of 12 different variants were detected in the patients, most of which were missense. Among these, 8 were pathogenic/likely pathogenic variants. Three variants classified as VUS have been previously reported in patients with PFIC. Three of these variants had not been previously reported in the literature and were considered novel. When the novel alterations were evaluated according to the ACMG criteria, one variant was classified as being of uncertain clinical significance and the other two as likely pathogenic and pathogenic variants. The novel frameshift variants p.Gln989Serfs*18 and p.Arg1249Serfs*39 in the

Table 1. General variant list and clinical findings

Patient No	Gender	Age	Gene (Transcript)	Variant	Variant type	Zygosity	HGMD/Novel	ACMG classification	Inherited from	Clinical findings
1	F	5 mos	*ATP8B1 (NM_005603)*	c.2854C > T (p.Arg952*)	Nonsense	Hom	CM043830	P	Parents	I, J, D, C, ALF, LT
2	F	3 mos	*ABCB11 (NM_003742)*	c.2842C > T (p.Arg948Cys)	Missense	Hom	CM081493	P	Parents	I, J, C, ALF
			ATP8B1 (NM_005603)	c.1798C > T (p.Arg600Trp)	Missense	Het	CM043820	LP	Mother	
3	M	2 yrs 2 mos	*ABCB11 (NM_003742)*	c.1160G > A (p.Arg387His)	Missense	Het	CM103533	VUS	Mother	C, H, S, GR
				c.3703C > T (p.Arg1235*)	Nonsense	Het	CM081481	P	Father	
4	M	7 yrs 7 mos	*ABCB4 (NM_000443)*	c.2858C > A (p.Ala953Asp)	Missense	Hom	CM055899	P	Parents	I, J, D, C, H
5	M	13 mos	*ABCB4 (NM_000443)*	c.2858C > A (p.Ala953Asp)	Missense	Hom	CM055899	P	Parents	I, J, C, H
6	M	5 yrs 6 mos	*ABCB11 (NM_003742)*	c.2708T>G (p.Val903Gly)	Missense	Hom	Jeyaraj *et al.* [16]	VUS	Parents	H, C
7A	F	1y	*ABCB11 (NM_003742)*	c.2636T > G (p.Ile879Arg)	Missense	Hom	Bakır *et al.* [22]	VUS	Parents	J, C, H, S, GR
7B	F	5 yrs 8 mos	*ABCB11 (NM_003742)*	c.2636T > G (p.Ile879Arg)	Missense	Hom	Bakır *et al.* [22]	VUS	Parents	J
8	F	3 mos	*ABCB4 (NM_000443)*	c.3100A > G (p.Ile1034Val)	Missense	Hom	Novel	VUS	Parents	I, J, C, H
9	M	2 mos	*ABCB11 (NM_003742)*	c.403G > A (p.Glu135Lys)	Missense	Het	CM092737	LP	Mother	I, J, C, ALF, LT
				c.2965delC (p.Gln989Serfs*18)	Frameshift	Het	Novel	P	Father	
			ABCB4 (NM_000443)	c.3768_3769delAG (p.Arg1249Serfs*39)	Frameshift	Het	Novel	LP	Father	

Variants significant for progressive familial intrahepatic cholestasis (PFIC) are listed with their clinical manifestations and characteristics of the variants. Variants reported in the literature are given with the HGMD number. Variants not reported in the literature are given by evaluating according to ACMG criteria.

F = female, M = male, HDMD = Human Gene Mutation Database, ACMG = American College of Medical Genetics and Genomics, VUS = variants of uncertain significance, LP = Likely pathogenic, P = Pathogenic, Hom = Homozygous, Het = Heterozygous, I = itching, J = jaundice, D = diarrhea, GR = growth retardation, ALF = acute liver failure, C = cholestasis, H = hepatomegaly, LT = liver transplant, S = splenomegaly.

ABCB11 gene, and p.Ile1034Val in the *ABCB4* gene were predicted as 'damaging' by the SIFT/PROVEAN and PolyPhen-2 web software.

DISCUSSION

Variants in genes encoding hepatobiliary transport proteins can cause a wide range of cholestatic liver diseases, from PFIC to milder forms with limited episodes of cholestasis. PFIC is classified into subgroups depending on clinical examination, laboratory findings, and genetic defect. This disease, classically divided into three types (1-3), is known to be caused by biallelic pathogenic variants of *ATP8B1* (encoding FIC1 protein), *ABCB11* (encoding BSEP protein), and *ABCB4* (encoding MDR3 protein) [9, 10].

PFIC is a disease diagnosed in childhood. It ranges from symptoms suggestive of cholestasis, such as pruritus and jaundice, to liver findings that can lead to cirrhosis and liver failure, vitamin K insufficiency, diarrhea, and developmental delays due to malabsorption. Extrahepatic findings such as pancreatitis and hearing loss may be detected, and severe FIC1 defects occur as early as the first year of life [11]. According to our list, we detected a homozygous *ATP8B1* nonsense variant previously reported in the literature only in a 5-month-old female patient. For the first time in the literature, the *ATP8B1* gene p.Arg952* variant was discovered as compound heterozygous in three different patients (two with PFIC and one with BRIC) by Klomp *et al*. [12]. This variant, which has not been reported in biallelic form in the literature, was homozygous in our patient and resulted in a severe clinical course [4]. Our patient, who complained of jaundice and diarrhea in the first month after birth, developed liver failure within a short time and had to undergo liver transplantation at the age of 2 years. In addition to PFIC1, she had congenital hypothyroidism and umbilical hernia.

In our study, we detected different variants associated with PFIC2 in 6 patients from 5 different families. The variant p.Gln989Serfs*18 detected in the *ABCB11* gene of patient 9 was novel. It is generally believed that PFIC2, also known as BSEP defect, shows rapid progression of hepatic fibrosis [13]. Although we do not have sufficient data to support this finding, we see patients referred to our laboratory for molecular genetic analysis within the first five years of life. Indeed, the diagnoses of patients 2 and 9 were confirmed by molecular genetic analysis within a few months of birth. Vitale *et al*. detected the variant p.Glu135Lys on the *ABCB11* gene in a 16-year-old male patient as compound heterozygous with another pathogenic frameshift variant. The patient's liver symptoms, which began as neonatal jaundice and itching, progressed to failure requiring transplantation [14]. We detected the p.Glu135Lys variant as a compound heterozygote with the p.Gln989Serfs*18 variant, which was classified as possibly pathogenic according to ACMG criteria, in our 2-month-old male patient who had similar clinical findings to this case. Liver transplantation was performed in our patient, who rapidly developed liver failure at seven months of age. Moreover, in addition to the alterations causing PFIC2, we incidentally detected a likely pathogenic heterozygous alteration in the *ABCB4* gene. It is complicated to comment on the effects of this alteration, which he inherited from his healthy father and which has not been reported in the literature with respect to the patient's clinical findings. Similarly, we detected an incidental variant in patient 2. A heterozygous *ATP8B1* variant was detected together with a homozygous *ABCB11* variant causing PFIC2. It has been reported in the literature that heterozygous variant of the *ATP8B1, ABCB11*, and *ABCB4* genes can cause mild forms of cholestatic liver disease. However, as in patients 2 and 9, there are insufficient data in the literature on the effects of other incidental heterozygous alterations detected in addition to those causing the actual PFIC clinical picture on the clinical findings of patients [15]. This situation will become more precise as the number of patients and functional studies of the detected variants increase. Patient 6 with the homozygous c.2708T > G variant had hepatomegaly and cholestasis findings. Patients with the same variant reported by Jeyaraj *et al*. [16] had acute liver failure in addition to the findings in our patient. Patients 3 and 7A also had cholestasis and hepatosplenomegaly and developmental delay. Patient 7B, the sibling of patient 7A, had no other findings except mild jaundice. It has been reported in the literature that different phenotypes are observed in siblings with the same genotype. The presence of genetic, epigenetic, or environmental modifiers could partially explain the different expression of the disease in family

members with the same homozygous gene variant. The literature reports that splicing and frameshift variants in the *ATP8B1, ABCB11,* and *ABCB4* genes are usually associated with severe disease, whereas missense variants are associated with less severe disease. In our patient cohort, there were insufficient variants to evaluate these data [17, 18].

We detected homozygous *ABCB4* variants associated with PFIC3 in three cases from different families. Although there is no association between them, we found the same *ABCB4* variants in patients 4 and 5. In patient 8, we detected a novel homozygous variant of uncertain clinical significance (according to ACMG criteria). In contrast to the literature, our patients had no other characteristics besides the findings of cholestasis. Unlike other groups, PFIC3 may show an insidious onset. Biochemically, it is associated with high GGT, and gallstones are a common USG finding. The development of hepatocellular carcinoma and cholangiocarcinoma is more common in PFIC3 patients [19].

We found a homozygous c.616A > G alteration in the *ABCB11* gene in our 13-month-old male patient who is not on the list. Although this alteration is considered benign, it has not been reported in the homozygous form in healthy individuals in population studies (gnomAD; genome aggregation database). The fact that our case with cholestasis findings was homozygous for this alteration suggested PFIC2 as a preliminary diagnosis, but her healthy 5-year-old sister was also homozygous for this alteration. This situation showed us once again the importance of segregation analysis.

It has been reported in the literature that panel-based NGS is a very useful tool for the diagnosis of cholestatic liver disease when extrahepatic causes have been excluded [20, 21]. In our study, a molecular genetic diagnosis was made in 10 of 37 patients whose PFIC gene panel was examined, representing a diagnostic yield of 27%. In a similar study recently conducted by Bakır *et al*., the diagnostic yield was found to be 40% [22]. Moreover, in a substantial number of patients with the PFIC phenotype, no variants can be identified in the *ATP8B1, ABCB11,* and *ABCB4* genes. Some of these cases are known to be associated with variants in other genes involved in the secretion of bile salts [23]. Considering that there are currently 12 subtypes of PFIC and other genetic diseases that cause cholestasis, future efforts will be made to achieve a higher diagnostic rate to determine the molecular background of cholestatic liver disease by adding new genes to the panel gene content we used.

CONCLUSION

In this study, we evaluated the results of molecular genetic analysis and clinical findings of patients who underwent NGS study with the gene panel covering the most common PFIC subtypes. Three variants considered novel have been presented in the literature. The beneficial clinical use of NGS-based genetic panels has been demonstrated in cases where PFIC is clinically suspected.

Authors' Contribution

Study Conception: AT; Study Design: AT; Supervision: AT; Funding: AT; Materials: AT; Data Collection and/or Processing: AT; Statistical Analysis and/or Data Interpretation: AT; Literature Review: AT; Manuscript Preparation: AT and Critical Review: AT.

Conflict of interest

The author disclosed no conflict of interest during the preparation or publication of this manuscript.

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