ABCB4 disease mimicking morbus Wilson: A potential diagnostic pitfall

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Introduction. Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare autosomal recessive cholestatic liver disorder caused by genetic deficiency of ATP-binding cassette subfamily B member 4 (ABCB4), a hepatocanalicular floppase translocating phospholipids from the inner to the outer leaflet of the canalicular membrane lipid bilayer. PFIC3 is characterised by production of hydrophilic bile with lithogenic properties which is harmful to the hepatobiliary epithelia. Chronic cholestasis in some patients may be accompanied by excessive accumulation of copper in the liver and by increased urinary copper excretion, the findings mimicking Wilson disease (WD).
Methods and Results. We report an 11 y/o male patient with growth retardation, mild craniofacial dysmorphic features and chronic liver disease, initially diagnosed and treated as WD. Whereas genetic testing for WD was negative, further molecular and histopathological analysis revealed two novel mutations (c.833+1G>T and c.1798T>A) in *ABCB4* and complete absence of the ABCB4/MDR3 protein in the liver, determining PFIC3 as the correct diagnosis.
Conclusion. PFIC3 and WD display pleomorphic and sometimes overlapping clinical and laboratory features, which may pose a differential diagnostic problem. Since the patient management in WD and PFIC3 differs significantly, an early and accurate diagnosis is crucial for optimising of therapeutic approach and prevention of possible complications.

Key words: ABCB4, progressive familial intrahepatic cholestasis type 3, Wilson disease, copper metabolism, ATP7B

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INTRODUCTION

Progressive familial intrahepatic cholestasis type 3 (PFIC3, OMIM #602347, gene *ABCB4*) is an autosomal recessive cholestatic liver disorder caused by absence of functional multidrug resistance protein 3 (MDR3), also known as ATP-binding cassette (ABC) subfamily B member 4 (ABCB4) (ref.[1]). ABCB4/MDR3 is a hepatocanalicular transporter, "floppase", translocating phosphatidylcholine from the inner leaflet to the outer leaflet of the canalicular membrane lipid bilayer[2-4]. Most of the harmful effects of *ABCB4* deficiency on hepatobiliary system are attributable to "toxic bile" with potent detergent and lithogenic properties. Typical laboratory findings associated with PFIC3 are characterised by predominantly conjugated hyperbilirubinemia and elevated serum γ-glutamyl transferase (GGT) activity[1]. Moreover, chronic cholestasis may be accompanied by increased urinary copper excretion and significant accumulation of copper in the liver, i.e., findings that may be potentially misinterpreted as Wilson disease (WD, gene *ATP7B*) (ref.[5-8]).

In this report, we describe the case of an 11 years old male patient suffering from chronic liver disease with biochemical parameters indicating impairment of copper metabolism and excessive copper accumulation in the liver typical for WD. However, subsequent molecular genetic and histopathological analysis revealed mutations in *ABCB4* and complete absence of canalicular ABCB4/MDR3 protein expression.

CASE REPORT

An 11-year-old male patient was initially referred to the local hospital due to growth retardation and a marked hepatosplenomegaly detected during his periodic preventive health screening. The boy complained of occasional pains in the right upper abdominal quadrant and intermittent pruritus. There was no history of bleeding or significant fatigue and his psychomotor development was normal. His family history was unremarkable in terms of hereditary disorders or liver diseases.

Physical examination revealed hepatomegaly with the enlarged firm liver (+ 10 cm at the midclavicular line) and splenomegaly (+ 8 cm). Moreover, marked hirsutism, a short statue (body height 121.5 cm, 4 SD below the

mean), and mild craniofacial dysmorphia with dolicho-cephaly and a flattened face, the features not attributable to familial factors, were also of notice.

Laboratory examinations were notable for pathological liver function tests with elevated serum GGT (5.5 µkat/L), alanine aminotransferase (ALT, 1.9 – 7.0 µkat/L), and aspartate aminotransferase (AST, 1.8 – 11.0 µkat/L) activity. Total bilirubin level was 53 - 75 µmol/L with conjugated bilirubin level between 42 and 52 µmol/L. Serum ceruloplasmin, α-1-antitrypsin, and albumin, as well as serum lipid levels were initially within the normal range, while urinary copper output was increased to 178 µg/24h. Serological tests for viral hepatitis, autoantibodies, and endocrine screening were negative. Metabolic screening excluded inborn errors of iron, porphyrin, amino acid, and lipid metabolism. As a part of the diagnostic process, core liver biopsy was performed. Advanced septal fibrosis with periportal ductular reaction and hepatocanalicular cholestasis was found, with copper level of 914 µg/g dry weight. Based on these findings, WD was entertained as the most likely diagnosis and treatment with metalcaptase was initiated. However, subsequent slit lamp examination showed absence of Kayser-Fleischer ring and mutational analysis did not reveal mutations in *ATP7B*, thus calling the diagnosis of WD into question.

Within a few months, the liver disease progressed, along with manifestation of portal hypertension and further deterioration of laboratory parameters: total bilirubin level 99.1 µmol/L (conjugated bilirubin 60.5 µmol/L), GGT 5.77 µkat/L, ALT 17.0 µkat/L, AST 19.8 µkat/L, albumin 32.3 g/L, triacylglycerols 3.1 mmol/L, cholesterol 3.3 mmol/L, ammonia 53.6 µmol/L, and α-fetoprotein 12.4 µg/L. Blood count and coagulation parameters were as follows: haemoglobin 133 g/L, haematocrit 0.402, red blood cell count 4.51×10^{12}/L, white blood cell count 3.3×10^9/L, platelet count 62×10^9/L, APTT 36.2 s, prothrombin time (PT)-Quick 19.60 s, PT-ratio 1.4, INR 1.71, fibrinogen 2.11 g/L, and antithrombin 54%.

Abdominal ultrasonography and CT scan demonstrated hepatomegaly with several parenchymal nodules up to 15 mm, marked splenomegaly, ascites and portosystemic collateral circulation.

Due to gradual progression of the liver disease with unsatisfactory response to the conservative therapy, the patient was referred to the transplant centre, where he underwent split-liver transplantation.

The liver explant removed at transplantation showed diffuse nodularity of the parenchyma with a green discoloration of the nodules. The liver tissue was fixed in 4% paraformaldehyde and processed for histological examination. Sections cut at 4-6 µm were stained with hematoxylin and eosin, periodic acid-Schiff-diastase reaction, orcein and elastin-van Gieson's method. For immunohistochemical analysis, sections were incubated with the anti-MDR3/ABCB4 rabbit polyclonal antibody (NBP2-30887PEP, Novus Biologicals, Colorado). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualisation and counterstaining with Harris's hematoxylin was performed. Light microscopy revealed predominantly micronodular biliary cirrhosis with a mild residual inflammatory infiltrate within the portal tracts and septa. Moreover, features of chronic cholangiopathy with periportal ductular reaction, as well as morphology of cholestasis with cholestatic liver cell rosettes, bile infarcts, Denk-Mallory hyaline inclusions and copper-associated protein depositions in juxtaportal hepatocytes were also easily discernible. In keeping with CT scan findings, the regenerative nodules up to 15 mm were present in the liver parenchyma but no malignancy was identified. Importantly, immunohistological examination demonstrated complete absence of hepatocanalicular ABCB4/ MDR3 expression in the patient's liver tissue (Fig. 1). In accordance with previous findings, excessive accumulation of copper with a level of 1867µg/g dry weight was demonstrated in the liver explant; however, based on the histopathological findings, diagnosis of PFIC3 was suggested.
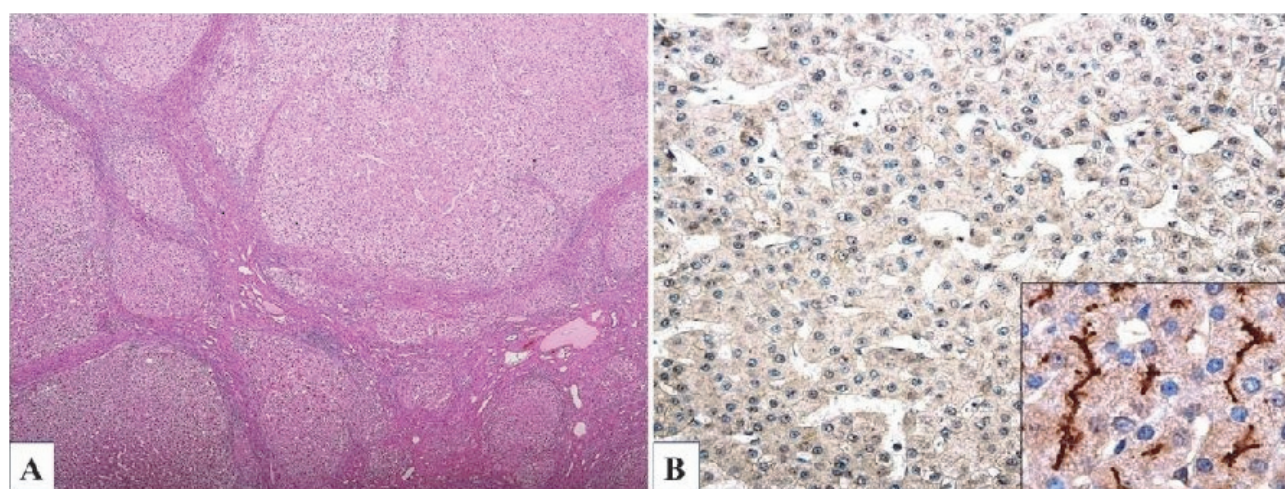

Fig. 1. Histopathology findings in the explanted liver.
(A) Haematoxylin and eosin, original magnification x40, (B) MDR3 immunohistochemistry, original magnification x200 (inset, x400).
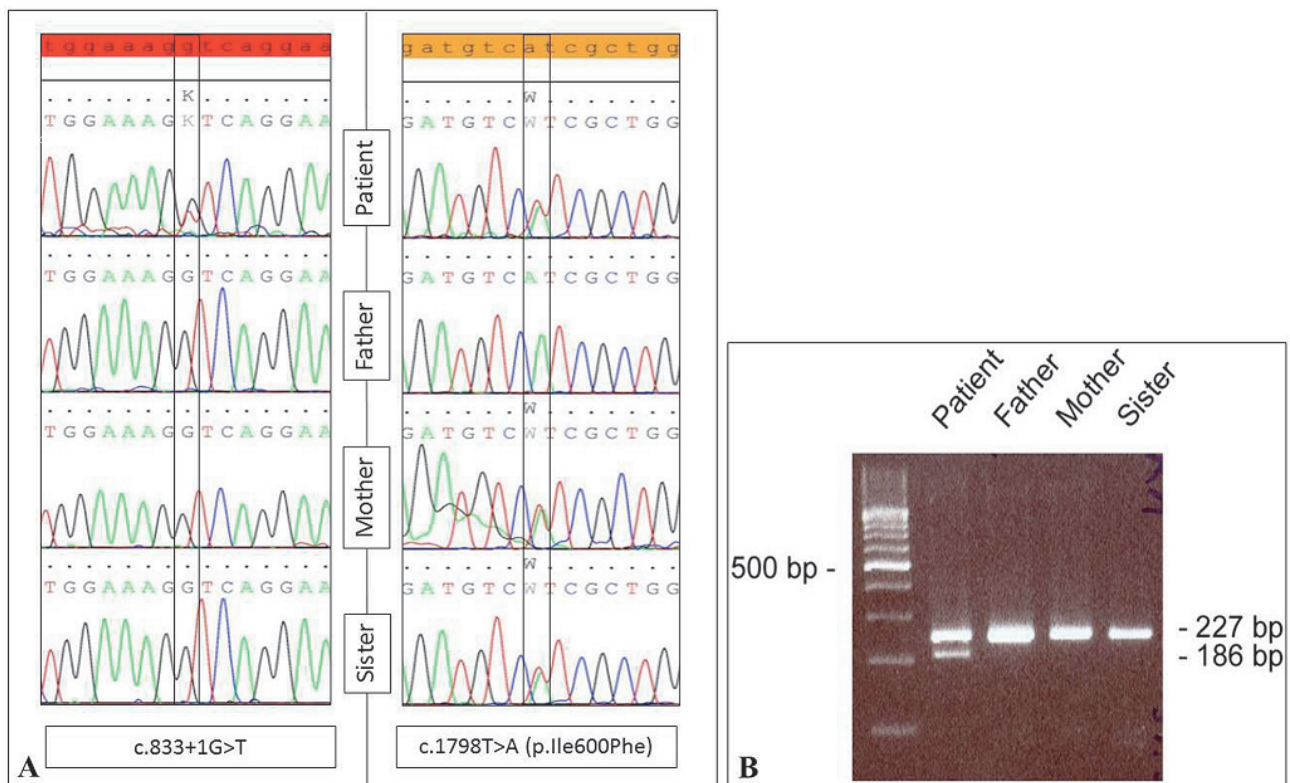
Fig. 2. Family analysis.
(A) Mutations found in the index patient and his first-degree relatives. (B) Detection of the c.833+1G>T mutation using PCR- *Hpy*8I restriction fragment length polymorphism analysis.

Targeted resequencing of *ABCB4* was then indicated to confirm the histopathological diagnosis of PFIC3. Written informed consent was obtained from the patient's legal representatives before genetic testing. *ABCB4* was analysed by direct sequencing of genomic DNA extracted from peripheral leukocytes on an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA). Two novel mutations c.833+1G>T and c.1798T>A in *ABCB4* were detected (Fig. 2).

Whereas pathogenicity of the former splice site mutation is apparent, pathogenicity of the latter, supposed to cause the amino acid substitution p.Ile600Phe, was tested *in silico* using PredictSNP1 (ref.[9]). The substitution located in the first ATP-binding cytoplasmic loop was rated as likely pathogenic with the overall pathogenicity score 87%. Both mutations were detected in heterozygous state. Family analysis revealed that the patient's mother and sister are heterozygous carriers of the mutation c.1798T>A but none of them carries the mutation c.833+1G>T. The father was shown to be free of both mutations. Therefore, we designed a PCR- *Hpy*8I RFLP method using a combination of a standard forward primer extended with the T7 overhang (5´-taatacgactcac-tatagCAGAGTGCCTTTAAACTTTTCTCC-3´) and a mutated reversed primer extended with the RP overhang (5´-tgaaacagctatgaccatgAGTGGCTAAAGAACCTTC-gTG-3´, both overhangs are *in italics*) and confirmed that the mutation c.833+1G>T was present exclusively in the index patient (Fig. 2B).

Segregation of both mutations in the family, together with complete absence of MDR3 protein in the liver, strongly suggests that both mutations detected in the proband are located in *trans*. Absence of the splice site mutation in the patient´s father can have multiple explanations including *de novo* mutagenesis or gonadal mosaicism.

DISCUSSION

WD (OMIM #277900) is an autosomal recessive disease caused by mutations targeting the copper transporter ATP7B, characterised by organ copper build up due to impaired copper metabolism and biliary secretion. Clinical manifestations range from asymptomatic course to variable combination of neuropsychiatric and hepatic symptoms including life-threatening liver failure. The diagnosis of WD is based on clinical and biochemical findings, excessive liver copper accumulation and molecular genetic analysis[10-12]. Similarly to WD, PFIC3, caused by almost complete biallelic inactivation of *ABCB4*, also displays pleomorphic clinical and laboratory phenotype, severity of which is affected at least partially by the *ABCB4* allelic status[13-15]. The disease usually occurs in early childhood and is characterised by hepatosplenomegaly with jaundice, pruritus, steatorrhea, and growth retardation[1].

Since liver disease manifests in both WD and PFIC3, differential diagnosis can be complicated. Whereas extrahepatic manifestations such as Kayser-Fleischer ring,

Table 1. Published patients with PFIC3 mimicking Wilson disease.

Age	Sex	Liver copper content (µg/g)	Urinary copper output (µg/24 h)	Serum ceruloplasmin (g/L)	Serum copper conc. (µg/L)	GGT (IU/L)	ABCB4 mutations	Ref.
21	M	NA	1733	0.14	NA	83	NA	8
15	F	1471	342	0.38	1510	734	c.3218G>A (p.C1073Y) c.984T>G (p.Y328*)	7
11	F	860	125	0.27	840	293	c.2563C>T (p.Q855*) c.1283T>C (p.V428A)	6
7	M	863	66	0.45	NA	608	c.490T>G (p.W164G) c.3081+1G>C	6
2	F	248	NA	0.28	1013	405	p.A546D, p.R176W	5

M – *male*, F – *female,* NA – not assessed.

Reference range: liver copper content <250 µg/g dry weight, urinary copper output <60 µg/24 h, serum ceruloplasmin 0.2 – 0.5 g/L, serum copper 750 – 1450 µg/L.

renal tubular acidosis or cardiomyopathy point to WD, growth retardation is more likely linked with chronic cholestasis. Unfortunately, the short statue of our patient was partly explicable by familial factors (mother's height 148 cm, father's height 162 cm). Interestingly, the patient also displayed marked hirsutism and a craniofacial dysmorphia, the features that have not been reported in PFIC3 patients.

The liver plays a central role in copper metabolism, being responsible predominantly for its storage and biliary excretion. Quantification of hepatic copper content provides valuable information for differential diagnosis, and severe copper accumulation is a strong indicator of WD. However, elevated concentrations of copper in the liver tissue as well as increased urinary copper excretion have also been reported in liver disorders not related to WD, especially in patients with prolonged cholestasis[12]. We found only four reports[5-8] documenting abnormal or marginal hepatic copper content in five patients with PFIC3 mimicking WD (Table 1). Four patients had elevated urinary copper excretion, one had also decreased serum ceruloplasmin level. To the best of our knowledge, the value of hepatic copper content 1867 µg/g dry weight on the background of *ABCB4* deficiency is the highest recorded so far (Table 1).

Since histopathological findings associated with both WD and PFIC3 vary widely, sometimes exhibiting morphological overlap, light microscopic analysis of the liver tissue is usually of limited diagnostic importance. However, immunohistochemical evidence of significant reduction or complete absence of the hepatocanalicular MDR3 protein expression, as was demonstrated in our case, may serve as a beneficial but not completely reliable diagnostic tool[16].

Untreated WD and PFIC3 are generally progressive and lethal. Unlike PFIC3, in which patients profit predominantly from administration of ursodeoxycholic acid, WD therapy is based on drugs increasing urinary copper elimination (chelators) or reducing intestinal copper absorption (zinc salts). Interestingly, no beneficial effect of chelation therapy on disease progression has been observed in PFIC3 or other non-WD chronic cholestatic diseases. Liver transplantation remains the ultimate treatment modality for both diseases, especially for rapidly progressive forms and/or for end-stage liver disease[10,11,17].

In summary, PFIC3 and WD display pleomorphic and sometimes overlapping clinical and laboratory features, which may pose a differential diagnostic problem. Since patient management in WD and PFIC3 differs significantly, early and correct diagnosis is crucial for optimising the therapeutic approach, deceleration of pathological processes and prevention of possible complications.

ABBREVIATIONS

ABC, ATP-binding cassette subfamily; ABCB4, ATP-binding cassette, subfamily B, member 4; ATP7B, ATPase, copper transporting, beta polypeptide; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, γ-glutamyl transferase; MDR3, Multidrug resistance protein 3; OMIM, Electronic database Online Mendelian Inheritance in Man™; PFIC3, Progressive familial intrahepatic cholestasis type 3; WD, Wilson disease.

Author contributions: ES, MJ: designed the study and wrote the manuscript; ES: performed histochemical and immunohistological analysis; MN, MJ: carried out mutational analysis; RK: participated in collecting the laboratory and clinical data; IS: performed laboratory evaluation of copper metabolism; All authors revised and edited the draft and are in agreement with the content of the manuscript.

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