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GENETICKÉ FAKTORY V ETIOLOGII A PATOGENEZI CHOLESTÁZ S NÍZKOU HLADINOU GAMMAGLUTAMYLTRANSFERÁZY A DĚDIČNĚ PODMÍNĚNÝCH ŽLOUTENEK

GENETIC FACTORS IN AETIOLOGY AND PATHOGENESIS OF LOW GAMMA-GLUTAMYLTRANSFERASE CHOLESTASIS AND HEREDITARY JAUNDICE.

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Vedoucí závěrečné práce: Doc. MUDr. et Mgr. Milan Jirsa, CSc.

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Abstrakt

Identifikace genů kódujících hepatocelulární přenašeče a funkční charakterizace jednotlivých proteinů v posledním desetiletí umožnily vedle zrychlení a zpřesnění diagnostického procesu cholestázy i bližší studium regulace genové exprese a tím potenciální možnosti jejího ovlivnění. Tato práce ilustruje současný pokrok na poli geneticky podmíněných cholestáz a zaměřuje se na cholestázy s nízkou hladinou gammaglutamyltransferázy (γGT) a dědičně podmíněné žloutenky. Studie popisuje různé poruchy transportního systému hepatocytů, charakterizuje mutace a jejich fenotypické následky a rozšiřuje tyto analýzy o podrobnou charakterizaci regulačních oblastí genu *ATP8B1*.

Abstract

Recent progress in understanding the molecular mechanism of hepatobiliary disorders enabled the improvement of diagnostic accuracy and promoted the study of the regulation of gene expression and its potential modifying factors.

Current achievement in the field of genetically determined cholestatic disorders is well illustrated in this thesis, focused on low gamma-glutamyltransferase (γGT) cholestasis and hereditary jaundice. The study describes several distinct defects of hepatocyte transport system, characterises underlying mutations and their phenotypic consequences and, finally, extends these studies for detailed characterisation of *ATP8B1* gene regulatory regions.

Klíčová slova: cholestáza, žlučové kyseliny, hyperbilirubinémie

Keywords: cholestasis, bile acids, hyperbilirubinemia

Genetic factors in aetiology and pathogenesis of low gamma-glutamyltransferase cholestasis and hereditary jaundice.

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1. Introduction

1.1 General preface

The liver plays an essential role in many physiological processes including glucose homeostasis, urea metabolism, synthesis of plasma proteins, bile acids (BA) and 25-hydroxycholecalciferol, detoxification of various endogenous and exogenous substances, and bile formation. Bile is necessary for absorption of dietary lipids and fat soluble vitamins, elimination of toxic compounds and cholesterol homeostasis, as cholesterol can only be excreted via bile (1).

Bile is formed in the liver and between meals is stored in the gallbladder. It is released from the gallbladder in response to digestion of a meal and reaches the small intestine, where the main component of bile – bile salts, acts as an emulsifier of dietary lipids, fat soluble vitamins and cholesterol. Bile salts are efficiently reabsorbed, mostly in the ileum, the rest in the colon after transformation by intestinal bacteria. Enterohepatic recirculation is very efficient, during 6-10 cycles per day 95% of bile salts are reabsorbed and returned to the liver via the portal circulation. The remaining 5% lost via faeces are replaced by de novo synthesis.

Cholestasis is defined as disruption of bile flow and may occur in the liver (intrahepatic cholestasis) or within the biliary tree (extrahepatic cholestasis) (2). Both acquired and hereditary forms have numerous clinical consequences including jaundice, malabsorption and liver damage. Despite the fact that hereditary forms of cholestasis are rare diseases, manifesting typically during infancy or childhood, they have greatly advanced our understanding of the physiology of bile secretion and, through them, most of the key genetic factors essential for the maintenance of normal bile flow have recently been identified.

1.2 Physiology and pathophysiology of bile secretion

1.2.1 Bile acid synthesis

Bile acids (BA) are the driving force ensuring efficient bile flow. The “primary” bile acids, cholic (CA) and chenodeoxycholic (CDCA), are synthesised from cholesterol in the liver. The pathway of bile acid biosynthesis includes several steps and can be divided into three subpathways, the classical (neutral) leading to CA, the alternative (acidic) resulting in CDCA

and the Yamasaki shunt. Irrespective of the differences in step order between subpathways, the syntheses in general comprises modification of sterol ring (introduction of hydroxyl group(s) into position 7α and 12α, epimerisation of 3β hydroxyl), shortening and oxidation of the side chain followed by conjugation with glycine and taurine (reviewed in (3)). As the pH of bile is alkaline and bile contains significant amount of sodium and potassium, bile acids are actually in the form of bile salts (BS).

The Yamasaki pathway is of little importance in humans under physiological conditions. However, its significance increases during early infancy and during cholestatic conditions leading to production of lithocholic acid (LCA), formed normally by the action of intestinal bacteria.

CA is a trihydroxy-bile acid with hydroxyl group at position 3α, 7α and 12α, CDCA is a dihydroxy-bile acid with a hydroxyl group at positions 3α and 7α. Once CA and CDCA reach the intestine, they are subjects of further modifications. By the activity of intestinal bacteria they are deconjugated and dehydroxylated at position 7α and thus form "secondary" bile acids, deoxycholic acid (DCA; 3α, 12α) and lithocholic acid (LCA; 3α). Both primary and secondary bile acids undergo efficient reabsorption by passive diffusion (glyco-conjugates) or active sodium-dependent transport (tauro-conjugates) (4). In the liver they are recaptured from portal blood by active transporters localised at the basolateral membrane of hepatocytes, reconjugated with glycine and taurine and further transformed into "tertiary" bile acids (e.g. by sulphatation). Tertiary bile acids do not undergo enterohepatic recirculation and are excreted via faeces.

The rate limiting steps of bile acid synthesis are catalysed by HMG-CoA reductase (synthesis of cholesterol) and 7-alpha-hydroxylase (5). Elevated levels of cholesterol increase BA synthesis, opposite to high levels of BA or vitamin C deficiency that suppress the BA synthetic pathway.

1.2.2 Molecular mechanism of bile secretion

Generation of bile flow is an osmotic process depending primarily (70%) on the secretion of BA (bile-acid dependent bile flow). Secretion of organic anions, including bilirubin, represents bile-acid independent function. Water and electrolytes passively follow the osmotic and concentration gradient through tight junctions. A role for the water channel aquaporin 8 has also been suggested. The secretion of the main bile solutes - bile acids, phospholipids, cholesterol and organic anions (including bilirubin) is an active process mediated by the battery of ATP-

dependent transporters localised in the apical (canalicular) membrane of hepatocytes. Most of the transporters involved in bile formation have now been identified. **(Figure 2).**

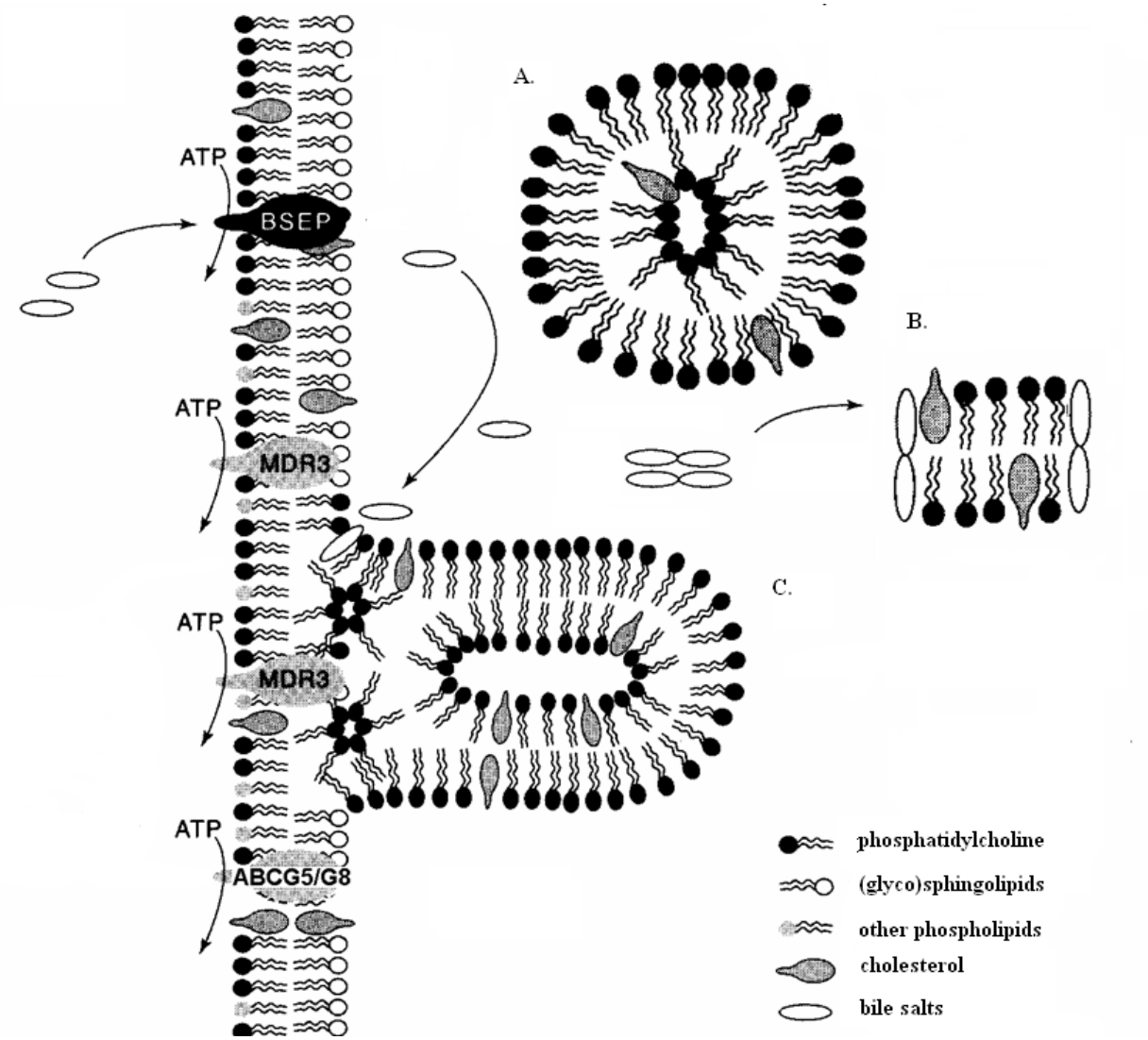


Figure 1: Two mechanisms of micelle formation are suggested. **(A.)** Phosphatidylcholine (PC) and cholesterol (CH) are actively secreted into bile canaliculi to form mixed vesicles. They serve as a source for micellar action of bile salt tetramers (simple micelles). Resulting mixed micelles **(B.)** are formed after bile salts exceed the critical micellar concentration. **(C.)** Another proposed mechanism supposes the membrane enrichment in PC and CH with their subsequent diffusion into canalicular bile and instant formation of mixed micelles (6, 7). The secretion of bile compounds is followed by their interactions within the canaliculi. Bile acids together with phosphatidylcholine (PC) form mixed micelles that serve as an acceptor for cholesterol (CH) (6, 8).

1.2.3 Cholestasis

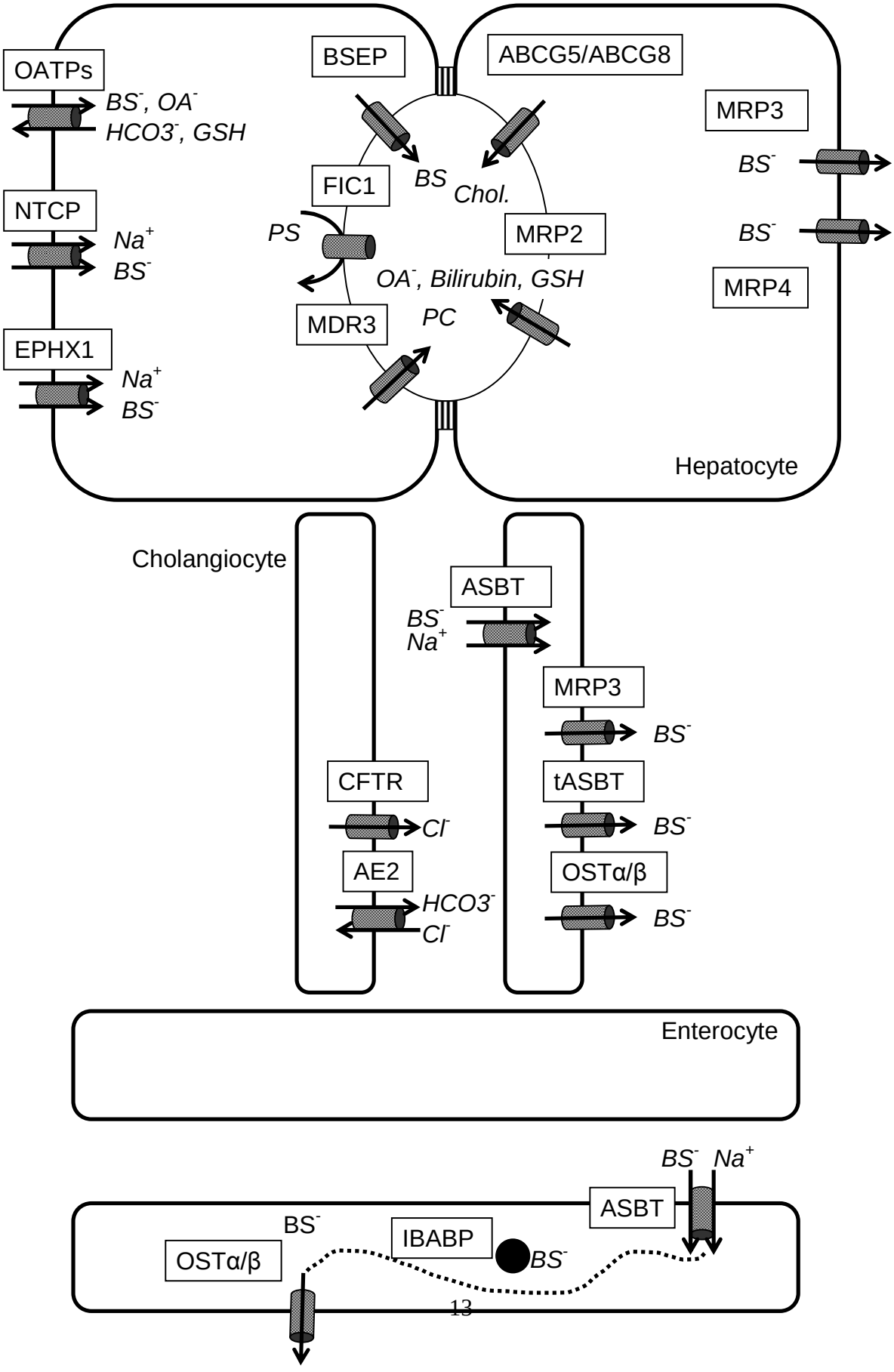
The amphipatic properties of BA enable formation of mixed micelles but are on the other hand responsible for the detergent effect of BA on biological membranes that is behind the BA cytotoxicity (6, 9). The cytotoxicity is well illustrated under cholestatic conditions in the liver where the prolonged exposure to BA results in membrane damage, cell injury and death, inflammation, fibrosis and eventually liver failure. Moreover, the disruption of bile flow also affects the secretion of other bile compounds, such as bilirubin, resulting in jaundice, and the lack of BA in the intestine severely impairs absorption of dietary lipids and fat-soluble vitamins, leading to steatorrhea and malnutrition (2).

Cholestasis clinically manifests with pruritus due to elevated serum bile salts and jaundice caused by hyperbilirubinemia. Malnutrition and vitamin K deficiency accompanied by failure to thrive in cholestatic children is other common feature (2, 10). Elevated serum gamma-glutamyltransferase (γGT) is considered a typical sign of cholestasis resulting from a prolonged exposure of canalicular membranes to a detergent effect of bile acids. However, a heterogeneous group of familial forms of intrahepatic cholestasis without elevated γGT exists. These disorders are characterised by impaired synthesis or secretion of BA into bile or by regurgitation of bile components due to a leaky biliary tree (for details see below) (11).

1.2.4 Transport proteins and their adaptive changes in cholestasis

Hepatocytes are polarised cells. Their apical (canalicular) and basal (sinusoidal) membranes are equipped with unique transport proteins that promote normal circulation of BS and other biliary compounds. Similarly, enterocytes posses various transporters enabling reabsorption of BS and thus contribute to their enterohepatic recirculation.

Figure 2: Hepatocellular, cholangiocellular and enterocyte transport proteins
NTCP: Na^+-taurocholate cotransporting polypeptide, OATPs: organic anion transporting polypeptides, EPHX1: microsomal epoxide hydrolase, MRP2: multidrug resistance elated protein 2, MDR3: multidrug resistance protein 3, MRP3: multidrug resistance protein 3, MRP4: multidrug resistance protein 4, BSEP: bile salt export pump, FIC1: familial intrahepatic cholestasis 1, ABCG5/ABCG8: ATP-binding cassette protein, subfammily G, member 5/member 8, CFTR: cystic fibrosis transmembrane conductance regulator, OSTα/β: organic solute transporter α/β, ASBT: apical sodium-dependent bile salt transporter, tASBT: truncated ASBT, IBABP: ileal bile acid-binding protein, AE2: chlorid-bicarbonate anion exchanger 2


OATPs
BS-, OA-
HCO3-, GSH
NTCP
Na+
BS-
EPHX1
Na+
BS-
BSEP
BS
Chol.
FIC1
PS
MRP2
OA-, Bilirubin, GSH
MDR3
PC
ABCG5/ABCG8
MRP3
BS-
BS-
MRP4
Hepatocyte
Cholangiocyte
ASBT
BS-
Na+
MRP3
BS-
CFTR
Cl-
tASBT
BS-
AE2
HCO3-
Cl-
OSTα/β
BS-
Enterocyte
BS-
Na+
ASBT
BS-
IBABP
OSTα/β
BS-

After their dissociation from albumin, BA are transported across the hepatocyte basolateral membrane in Na^+-dependent (NTCP, EPHX1) and Na^+-independent (OATPs) manners. NTCP (Na^+-taurocholate co-transporting polypeptide) represents the major bile salt uptake system of hepatocytes, whose driving force, the Na^+ concentration gradient, is maintained by Na^+/K^+ ATPase. NTCP transports both unconjugated and conjugated BA, the latter with a higher affinity (12).

The physiological significance of another Na^+-dependent transport system, microsomal epoxide hydrolase (EPHX1) isoform II, targeted to plasma membrane, remains to be elucidated.

The family of OATPs (organic anion transporting polypeptides) exchange unconjugated BA or extracellular organic anions with intracellular HCO_3^- and glutathione (GSH). OATPs work in an Na^+-independent manner and due to their broad substrate specificity are crucial players in clearance of organic compounds (including various drugs) clearance (13).

The bile acid secretory pathway is primarily directed into canaliculi, but hepatocytes also possess an alternative route activated in cholestasis. This route enables BA efflux across the basolateral membrane to protect liver cells against the toxic effect of BA. It is mediated by members of multidrug resistance proteins family MRP3 and MRP4 (14, 15) which are an ATP-dependent pumps with broad substrate specificity that includes conjugates of BA, glucuronic acid and glutathione.

Intracellular transport of BA is not yet fully understood. BA movement from sinusoidal to canalicular membrane of hepatocytes is driven by a concentration gradient and mediated by intracellular bile acid-binding proteins. The role of vesicle-mediated intracellular transport remains to be elucidated.

Canalicular secretion of monovalent BA is mediated by BSEP (bile salt export pump), whereas divalent sulphated or glucuronidated BA are transported via MRP2 (multidrug resistance related protein 2). MRP2, a major canalicular transporter responsible for bile salt independent bile flow, excretes a variety of organic compounds such as glutathione, sulphate and glucuronide conjugates including conjugated bilirubin.

The secretion of major biliary phospholipid, the phosphatidylcholine (PC), is mediated by the MDR3, which functions as a floppase, translocating PC from the inner to the outer leaflet of hepatocyte membrane. Inward flipping of aminophospholipids (phosphatidylserine, PS, and

phosphatidylethanolamine, PE) is an assumed function of FIC1 (familial intrahepatic cholestasis 1) protein. Its exact role in bile secretion is still not fully understood (for details see 3.1.4.).

Details of cholesterol transport are likewise complex and not fully explained. Heterodimer ABCG5/ABCG8, defective in patients with sitosterolemia, was originally shown to be a plant sterol transporter (16, 17) limiting the intestinal absorption and enhancing the liver excretion of sterols. Despite the experiments on mice deficient in abcg5 or/and abcg8 (18, 19) that demonstrated impaired ability to secrete cholesterol into bile and thus proved this transporter as a key player in cholesterol homeostasis, the exact mechanism by which cholesterol appears in bile is still not known. A liftase (rather than floppase) action of ABCG5/G8 was suggested by Ode Elferink and Paulusma (7), postulating that cholesterol push-up through the phospholipid bilayer made it available for the extraction by mixed micelles.

The **cholangiocyte transport system** plays a minor role under the physiological conditions, but during cholestasis it represents an important escape route for BS due to the 'cholehepatic shunt' that drives BS from the bile duct lumen directly into the circulation. Physiologically, most BA are transported as a part of mixed micelles in the intestine and only small quantities can be taken up by the apical sodium-dependent bile salt transporter (ASBT). Chloride entry into bile at the level of larger bile ducts is driven by cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) and bicarbonate secretion is driven by chloride-bicarbonate anion exchanger 2 (AE2). Bile acid secretion into the portal circulation by cholangiocytes is mediated as in the intestine, i.e. by multidrug resistance associated protein 3 (MRP3, ABCC3), OSTα/β or truncated ASBT (tASBT) (1, 20-22).

Intestinal bile salt transport. Most BS undergoe an enterohepatic recirculation and are reabsorbed in the terminal ileum by a sodium-dependent transport system. In addition to the most efficient ileal transporter, a sodium-independent jejunal system and passive diffusion of unconjugated BS within small and large intestine ensure that only as little as 5% of daily BA production is lost in the faeces.

The sodium-dependent transporter that shares 35% amino acid identity with NTCP, apical sodium-dependent bile salt transporter (ASBT), transports predominantly conjugated dihydroxy BS (23). Organic anion transporting polypeptides (OATPs) are probably responsible for sodium-independent intestinal transport of BS, even if final proof for their role in humans is missing (24, 25).

Intracellular BS transport within enterocytes is mediated by ileal BA-binding protein (I-BABP), which is on the cytoplasmic side of the apical enterocyte membrane, attached to ASBT (26, 27). Its substrate specificity includes beside BA also fatty acids, retinoids and cholesterol.

On the basis of rat studies, several candidate proteins were initially suggested as a part of the enterpcyte basolateral efflux system including tAsbt (Asbt splice variant with the features of anion exchanger overexpressed in rat ileum) (28) and Mrp3/MRP3 (expressed in human and rat intestine, particularly terminal ileum). However, the significance of these transporters is not clear and the main transport system is represented by the more recently discovered sodium-independent heteromeric organic solute transporter OSTα/OSTβ.

Adaptive changes of transport proteins in cholestasis. Synthesis, secretion and reabsorption of biliary lipids are highly controlled and regulated processes. The expression of transport proteins can be controlled at different levels by *trans*-acting and *cis*-acting elements. Despite significant progress in our understanding of regulation of gene/protein expression under physiological and pathological conditions, a complete explanation of the regulatory framework and its mutual relations still remains to be elucidated.

In the last decade interest was principally focused on transcriptional regulation and the role of nuclear receptors, partly because they represent the promising therapeutic targets that may enable control of lipid homeostasis. Once activated by various ligands, the nuclear receptors bind to specific DNA binding sites known as hormone responsive elements (HREs) and activate transcription of target genes. The HREs are usually organised as a direct, inverted or everted series of repeats separated by a variable number of nucleotides (usually n=1-6) and located in proximal promoters or distal enhancers of the gene (29). Primary bile acids (CA, CDCA) are potent regulatory ligands that act via the farnesoid X receptor (FXR). Once activated, FXR in turn inhibits BA uptake and stimulates BA efflux in both hepatocytes and enterocytes in order to prevent bile acid-induced cell damage (30-32).

Recent knowledge about the molecular mechanisms of cholestasis is based on animal model studies and, to a lesser extent, on observations of adaptive changes in human cholestatic diseases.

The gene/protein expression changes in cholestasis serve generally one main goal: To protect hepatocytes against the toxic action of BS. Hepatocytes can achieve this by reduction of BS entry across the basolateral membrane (FXR indirect downregulation of NTCP mediated by SHP-dependent block of RAR/RXR stimulating action exert on NTCP) and, at the same time, by

increasing BS export on the canalicular side (upregulation of BSEP) (33). BSEP is under the direct control of FXR/RXRα, which specifically bind bile acid response element (BARE) in the BSEP promoter and stimulate promoter activity (34). Additionally, MRP3, the basolateral hepatocyte and cholangiocyte transporter, also may be upregulated, in order to transport excess BS back into the circulation. Cholangiocytes react to cholestasis by proliferation and by upregulation of ASBT. They provide an escape route for excess BS from bile ducts to systemic circulation via basolateral membrane and MRP3.

Intestinal adaptive changes in cholestasis are rather obscure and conflicting results are published. ASBT expression in response to different BS pool seems species-specific. In humans, as with NTCP, the negative feedback regulation via an FXR-mediated, SHP-dependent effect was demonstrated (35). In accord with this finding, I-BABP and OSTα/OSTβ expression was proved to be under direct FXR control, with both genes up-regulated to prevent accumulation of BS in enterocytes (36).

1.3 Hepatocanalicular transport defects associated with cholestasis

Hand in hand with recent progress in understanding of the physiology of hepatocellular transport, increasing numbers of genetic diseases associated with cholestasis have been distinguished. Most hepatocanalicular transport defects are rare, infant-onset diseases, typically manifesting as neonatal cholestasis. Our better knowledge of the underlying molecular mechanisms has improved diagnostic accuracy and opens the possibility of future gene-targeted therapy.

1.3.1 *ATP8B1* deficiency

Gene: ATP8B1

Chromosome: 18q21

Protein: FIC1 (Familial Intrahepatic Cholestasis type 1)

- *function:* Unclear, thought to be an aminophospholipid flippase translocating phosphatidylserine from the outer to the inner leaflet of the canalicular membrane

Disease: PFIC1 (Progressive Familial Intrahepatic Cholestasis type 1), OMIM #211600, formerly Byler disease

Inheritance: Autosomal recessive

Phenotype: Highly variable phenotype ranging from progressive forms (leading to scarring of the liver) with onset in early infancy to milder, intermittent, non-progressive forms (often reffered as Benign Recurrent Intrahepatic Cholestasis, BRIC) (37). PFIC1 is characterised by jaundice, pruritus, coagulopathy and extrahepatic findings that include diarrhoea, failure to thrive, hearing problems, pancreatitis and abnormal sweat test. Aminotransferases (ALT, AST), alkaline phosphates (ALP) and bile acids (BA) are elevated, opposite to normal or low levels of γGT and cholesterol (2, 38). Low levels of γGT contrast with the elevated levels found in more frequent forms of neonatal cholestasis (e.g. biliary atresia) and represent an important diagnostic feature.

Light microscopy of the liver initially finds bland cholestasis without giant cell transformation, cellular infiltrate or bile duct proliferation. Progress to fibrosis and end-stage liver disease is typical. Upon transmission electron microscopy the bile has a coarsely granular appearance ('Byler bile') in contrast to the amorphous bile seen in other cholestatic conditions (39) (Fig. 2).

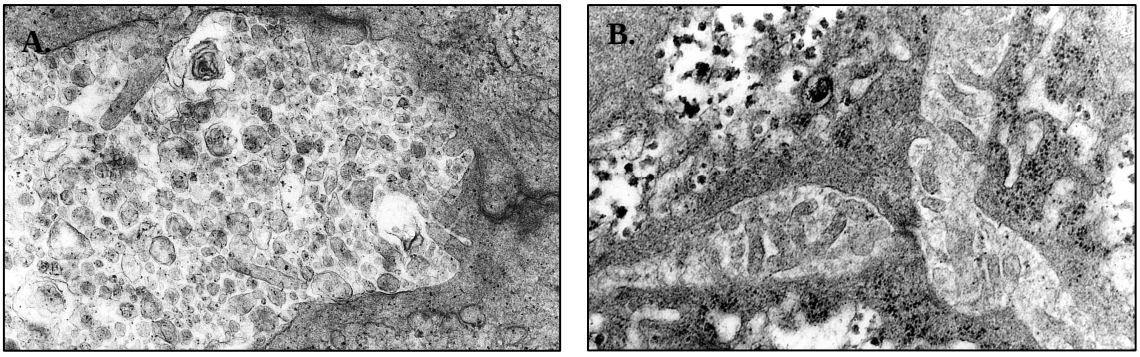


Fig. 2A. Dilated canaliculus with loose of microvilli filled with coarsely granular 'Byler' bile in *ATP8B1* deficiency. **2B.** Normal canaliculus. *Courtesy of Dr. Alex Knisely, Institute of Liver Studies, King's College Hospital, London.*

Immunohistological detection of FIC1 protein encounters technical difficulties and is not routinely used in a diagnostic assessment of *ATP8B1* deficiency. However, liver biopsies of PFIC1 patients may lack immunohistochemically detectable canalicular ectoenzymes such as γGT and CD10 (neutral endopeptidase) (40). Thus the investigation of ectoenzyme expression may be a beneficial tool in a diferentiating between *ATP8B1* and *ABCB11* deficiencies.

At the mild end of the spectrum of *ATP8B1* deficiency (BRIC) are the recurrent episodes of intrahepatic cholestasis with unpredictable onset and spontaneous resolution. Although trigger factors and effective therapy are unknown, temporary endoscopic nasobiliary drainage has

proved to be an efficient tool in decreasing pruritus and normalising serum bile acid concentrations (41). Histological findings demonstrate intrahepatic cholestasis during cholestatic episodes with complete recovery and no signs of permanent liver damage. However, patients in whom illness initially manifested in a recurrent form and later progressed to fibrosis are described. Although the severity and localisation of *ATP8B1* mutations might to a certain extent predict the clinical course of the disease (42), the phenotype of *ATP8B1* deficiency is actually a clinical continuum including both fatal and non-progressive presentations (43).

Current therapeutic strategies include cholecystojejunal cutaneous biliary diversion (partial biliary diversion; PBD) or ileal bypass. Reduction of the BA pool can lessen the accumulation of toxic secondary BA and thereby slow progression to end-stage liver disease. Transplantation is currently the only method able to resolve the liver disorder, but extrahepatic symptoms such as diarrhoea and failure to thrive tend to persist or, with diarrhoea, even to worsen.

The role of *ATP8B1* mutations in other cholestatic disorders

Greenland familial cholestasis, characterised by jaundice, bleeding, pruritus, malnutrition, steatorrhoea, osteodystrophy and dwarfism, occurs in indigenous Eskimo population (44) and was proved to be a part of the spectrum of *ATP8B1* deficiency. All affected individuals carry two copies of the same missense mutation: c.1660 G>A; p.Asp554Asn (45).

Intrahepatic cholestasis of pregnancy. Heterozygous mutations in *ATP8B1* were found in patients with intrahepatic cholestasis of pregnancy (46), suggesting *ATP8B1* haploinsufficiency as a possible predisposing factor.

1.3.2 *ABCB11* deficiency

Gene: ABCB11

Chromosome: 2q24

Protein: BSEP (Bile Salt Export Pump)

- *function:* Main canalicular bile salt transporter (tauroconjugates and glycoconjugates of CDCA and CA)

Disease: PFIC2 (Progressive Familial Intrahepatic Cholestasis type 2), OMIM #601847

Inheritance: Autosomal recessive

Phenotype: Similar to PFIC1 including low serum levels of γGT. The phenotypic spectrum includes progressive early-onset as well as remitting and late-onset phenotypes (47-49).

BSEP deficiency may present with gallstones and higher serum ALT and AST levels (corresponding to a more severe histological picture resulting from hepatocyte accumulation of toxic bile salts) (50). PFIC1 and 2 can be distinguished immunohistologically, genetically and sometimes ultrastructurally. On immunohistology, BSEP protein is absent in by far most cases of PFIC2 (48). On transmission electron microscopy, "Byler-type" loose, coarsely granular bile is characteristic of PFIC1 whereas amorphous or finely filamentous bile accompanied by giant cell transformation (as also seen on light microscopy) is typical for PFIC2. Patients with BSEP deficiency are at considerable risk of hepatobiliary malignancy (48, 51). Partial biliary diversion can offer clinical improvement in PFIC2 (52), but liver transplantation often is necessary for quality of life or survival. A subset of children with PFIC2 develop low γGT intrahepatic cholestasis after liver transplantation; this phenomenon has been traced to function-blocking antibodies against BSEP (53, 54). Treatment may be only partly successful (55).

1.3.3 *ABCB4* deficiency

Gene: ABCB4

Chromosome: 7q21

Protein: MDR3 (Multiple Drug Resistance Protein 3)

- *function:* Phosphatidylcholine (PC) floppase translocating PC from the inner to the outer leaflet of the canalicular membrane

Disease: PFIC3 (Progressive Familial Intrahepatic Cholestasis type 3), OMIM #602347

Inheritance: Autosomal recessive

Phenotype: Disease manifestations are determined by the impaired formation of mixed micelles and by loss of protection of canalicular and cholangiocyte membranes, as mediated by phosphatidylcholine secretion (7, 56), against effects of bile salts. Bile is more hydrophilic, but the lack of phospholipids in bile potentiates detergent properties and results in membrane damage. This leads to a progressive liver disease characterised by portal inflammation, proliferation of bile ducts and fibrosis.

Immunohistological detection of MDR3 protein is available and the lack of immunohistologically demonstrable MDR3 proves the diagnosis of PFIC3. γGT levels are high,

opposite to PFIC1 and 2. Treatment with UDCA is beneficial for some patients with MDR3 deficiency (57). Patients with progressive disease require liver transplantation.

Other phenotypic presentation of *ABCB4* deficiency:

LPAC (Low Phospholipid-Associated Cholelithiasis), OMIM #600803

ICP (Intrahepatic Cholestasis of Pregnancy), OMIM #147480

CIC (Oral Contraceptive-Induced Cholestasis)

Partial defects in MDR3 protein may manifest with a discrete phenotype that can be unmasked in later life, often in connection with extrinsic or intrinsic trigger factors. Intrahepatic Cholestasis of Pregnancy and Oral Contraceptive-Induced Cholestasis were described in patients with heterozygous mutations in *ABCB4* in response to hormonal load during pregnancy or during contraceptive pill treatment (58). The lack of phospholipids in bile of *ABCB4* deficient patients is responsible for decreased capacity of bile for cholesterol transport and thus results in cholesterol lithiasis known as Low Phospholipid-Associated Cholelithiasis (59, 60).

1.3.4 Defects in bile acid synthesis (BAS defects, BASD)

Genes: Various

Chromosomes: Various

Proteins, function: Enzymes of bile acid synthetic pathway

Disease: Various

Inheritance: Autosomal recessive

Phenotype: Variable, ranges from progressive liver disease with onset in early infancy to adult onset neuropathy or hyperlipidemia. Liver injury is likely caused by two mechanisms: accumulation of toxic bile acid intermediary metabolites and cholestatic action of reduced levels of normal primary bile acids (3). Diagnosis is based on serum and urine BA measurement and speciation using GC-MS (Gas Chromatography - Mass Spectroscopy) and FAB-MS (Fast Atom Bombardment - Mass Spectroscopy).

Whereas above mentioned PFIC1 and PFIC2 present with high levels of BA and low γGT, BASD is characterised by normal or low levels of serum BA together with low γGT. Substitution therapy with bile acid supplements is effective in most cases of BASD (61).

1.3.5 *CIRH1A* deficiency

Gene: CIRH1A, FLJ14728

Chromosome: 16q22

Protein: Cirhin

- *function:* Unknown

Disease: North American Indian childhood cirrhosis (NAIC); OMIM #604901

Inheritance: Autosomal recessive

Phenotype: Transient neonatal jaundice that resolves typically before the end of the first year (62). Progressive liver damage leads to biliary cirrhosis and portal hypertension, and liver transplantation is the only effective therapy. Biopsy shows early bile duct proliferation and rapid development of portal fibrosis. All affected individuals to date have carried a single mutation c.1741C>T (p.Arg565Trp) (63).

1.3.6 Familial hypercholanemia: Intestinal bile acid deficiency

Familial hypercholanemia is a heterogeneous group of disorders characterised by elevated serum BA and symptoms of BA deficit in the intestine. Efflux of BA from liver and plasma re-entry are associated with scant hepatocellular damage, although mild hepatitis and slight conjugated hyperbilirubinemia may be seen.

The disease mechanisms reflect the different underlying causes. Currently, defects in three unrelated genes *BAAT, TJP2* and *EPHX1* have been described in patients with FHCA.

Genes: BAAT, TJP2 (ZO-2), EPHX1

Chromosomes: 9q22-q32, 9q12-q13 and 1q42.1 respectively

Protein: Bile acid Coenzym A: amino acid N-acyl-transferase (BAAT), Tight-junction protein 2 (TJP2), Microsomal epoxide hydrolase (EPHX1)

- *function:*

- BAAT - conjugation of bile acids with taurin and glycine
- TJP2 - connection of tight junctions' transmembrane proteins to the actin cytoskeleton
- EPHX1 - sodium-dependent uptake of bile acids into hepatocytes

Disease: Familial hypercholanemia (FHCA); OMIM #607748

Inheritance: Autosomal recessive / complex

Phenotype: Cholestasis is a rare sign of FHC as the main symptoms result from deficiency of normal (conjugated) bile acids in the intestine: the disease is characterised by fat malabsorption leading to vitamin K dependent coagulopathy, rickets and failure to thrive. Itching due to high levels of serum bile acids is a frequent, but not constant symptom. Liver enzymes are normal except for ALP, serum BA are usually high or fluctuate, and γGT is normal. The patients respond well to therapy with ursodeoxycholic acid (UDCA) and general prognosis is supposed to be excellent, although there are no data from adults.

The underlying mechanism is the escape of bile acids from their normal route through the liver due to:

1) defect of conjugation with taurine and glycine that prevents normal canalicular secretion and results in diffusion of unconjugated BA through the basolateral membrane back into plasma (BAAT deficiency)
2) leakage of BA through defective tight junctions (TJP2)
3) impaired sinusoidal influx of BA into hepatocytes (EPHX1)

Incomplete penetrance and possible roles of other genes complicate clinical assessment of inheritance, as clinically unaffected siblings may be homozygous for TJP2 mutations (64). Interestingly, some FHCA patients homozygous for TJP2 mutations were also heterozygous for a particular BAAT mutation.

The role of *EPXH1* mutations in FHCA remains to be elucidated. The changes found in the promoter and the first intron of *EPHX1* in a single patient with the hypercholanemic phenotype were also detected in a small number of control chromosomes, suggesting that the changes might have a modifying rather then a causative effect in FHCA (65).

1.3.7 *CLDN1* deficiency

Gene: CLDN1

Chromosome: 3q28-q29

Protein: Claudin-1

- *function:* Structural tight-junction protein

Disease: Neonatal sclerosing cholangitis associated with ichthyosis (NISCH syndrome) or (ILVASC: Ichthyosis, Leukocyte vacuoles, Alopecia, Sclerosing Cholangitis); OMIM #607626

Inheritance: Autosomal recessive

Phenotype: Patients present with jaundice, pruritus, hepatomegaly, ichthyosis, scalp hypotrichosis and scarring alopecia. Other features include dysplastic enamel and intracytoplasmic vacuoles in eosinophils, although this last feature is not uniformly present (66). Histology shows progression to sclerosing cholangitis. Lack of CLDN1 expression in liver and keratinocytes leads to dysfunctional junctional complex and thus to the diffusion of solutes through the paracellular space (67, 68).

1.3.8 *VPS33B/VIPAR* deficiency

Genes: VPS33B, VIPAR

Chromosomes: 15q26.1 and 14q24.3 respectively

Proteins: VPS33B (Vacuolar Protein Sorting 33) and VIPAR (VPS33B-Interacting Protein; Apical-basolateral polarity Regulator)

- *function:* Intracellular trafficking and subsequent membrane fusion

Diseases: Arthrogryposis, Renal dysfunction and Cholestasis (ARC) syndromes 1 and 2 respectively; OMIM #208085 and OMIM #613404

Inheritance: Autosomal recessive

Phenotype: Multisystem disorders characterised by arthrogryposis multiplex congenita, renal tubular dysfunction, neonatal low γGT cholestasis and platelet dysfunction (69). Affected infants die within the first year of life. An incomplete phenotype (e.g. without arthrogryposis) has also been noted (70).

VIPAR was found to be mutated in patients with ARC who had no mutations in VPS33B. (71)

1.3.9 Notch signalling pathway defects

Genes: Jagged1 (JAG1), NOTCH2

Chromosomes: 20p12 and *1p13-p11* respectively

Proteins: JAG1 and NOTCH2 respectively

- *function:* Ligand and receptor respectively in Notch signalling pathway; exact function in bile duct development unknown

Diseases: Alagille syndrome (AGS) type I and type II respectively; OMIM #118450, (72, 73) and OMIM #610205 (74)

Inheritance: Autosomal dominant with highly variable expressivity

Phenotype: Developmental multisystem disorder. The paucity of small bile ducts is an underlying defect resulting in neonatal cholestasis, hypercholesterolemia and pruritus. The other common clinical findings include congenital heart disease (usually peripheral pulmonary stenosis), typical facies, skeletal malformations ('butterfly' vertebrae), posterior embryotoxon of the eye, renal and vascular abnormalities (75). Histologically AGS is characterised by loss of bile ducts in postnatal development, termed hypoplasia (76).

Despite the autosomal-dominant transmission, clinical presentation differs greatly within the same family, even between homozygote twins and the diagnosis is thus based on liver biopsy and genetic analysis.

1.3.10 Aagenaes syndrome, Lymphedema - cholestasis syndrome, LCS1

Gene: unknown

Chromosome: 15q

Protein: unknown

- *function:* unknown

Disease: Lymphedema-cholestasis syndrome

Inheritance: Autosomal recessive

Phenotype: Aagenaes syndrome is a very rare disease still awaiting the discovery of underlying genetic defect. The inheritance is autosomal recessive, even in some cases also another mode of transmission was suggested (77). The disease locus was mapped to chromosome 15q (78). Episodes of cholestasis begin in infancy; lymphedema worsens and "ascends" with age, progressing from pedal to truncal involvement. The liver disease manifestations may be intermittent or, with scarring, may require liver transplantation for survival (79).

1.4 Hepatocanalicular transport defects associated with hyperbilirubinemia

1.4.1 *ABCC2* deficiency (Dubin-Johnson syndrome)

Gene: ABCC2

Chromosome: 10q24

Protein: MRP2 (Multidrug resistance protein 2), formerly canalicular multispecific organic anion transporter (cMOAT)

- *function:* Transport of organic anions and neutral compounds

Disease: Dubin-Johnson syndrome (DJS); OMIM #237500

Inheritance: Autosomal recessive

Phenotype: Chronic conjugated hyperbilirubinemia without clinical-biochemistry evidence of hepatocellular injury and accumulation of brown, coarsely-granular, melanin-like pigment in hepatocytes (80, 81). Patients exhibit prolonged excretion of bromsulphthalein and delayed visualisation of the gallbladder on cholescintigraphy. Total urinary coproporphyrin levels are normal, but isomer I is markedly increased (in homozygotes over 80%) and as such represent useful indicator of DJS (82).

In most patients, ABCC2 protein is not immunohistologically detectable in the canalicular membrane; only a few express nonfunctional protein (83-85).

1.4.2 Rotor syndrome

Gene: Unknown

Chromosome: Unknown

Protein: Unknown

- *function:* Unknown

Disease: Rotor syndrome

Inheritance: Autosomal recessive

Phenotype: A rare disorder characterised by predominantly conjugated hyperbilirubinemia of unknown aetiology. Rotor syndrome is clinically similar to DJS, but without liver pigmentation (86, 87). Total coproporphyrin excretion is also increased with predominance of isomer I, but, unlike DJS, the proportion of isomer I does not reach 80%. Despite similar clinical presentations, Rotor syndrome is not an allelic variant of DJS (88).

2. Aims

This thesis aimed to address the role of genetic factors in the aetiology of low γGT cholestases and hereditary jaundice. Hence we focused primarily on *ATP8B1*, *ABCB11* and *ABCC2* genes, which encode the aminophospholipid flippase, the bile salt export pump and a transporter for conjugated bilirubin respectively.

Our objectives were:

1. To search for mutations in *ATP8B1* and *ABCB11* deficient patients and evaluate genotype-phenotype correlations
2. To assess the effect of specific *ABCB11* genotypes on expression of immunohistochemically detectable BSEP protein and to evaluate the potential role of severe *ABCB11* mutations in development of hepatobiliary malignancy
3. To determine the unknown regulatory regions responsible for transcriptional control of *ATP8B1* gene that could be potentially affected in FIC1 deficient patients in whom no mutation in *ATP8B1* coding region was found
4. To characterise the role of *ABCC2* in a mixed, previously undefined type of hyperbilirubinemia and its putative role in a Rotor-type conjugated jaundice

3. Results and commentary

3.1 Low gamma-glutamyltransferase cholestasis

3.1.1 Mutational analysis of the *ATP8B1* and *ABCB11* genes in Czech patients with mild phenotype (formerly Benign Recurrent Intrahepatic Cholestasis type 1 and 2 – BRIC1 and BRIC2)

Analysis of three Czech individuals identified the following defects:

Proband 1: c.1982T>C (p.Ile661Thr) and **c.3122del5/ins20** in *ATP8B1*

Proband 2: **c.403G>A** (Glu135Lys) and **c.1757C>T** (p.Thr586Ile) in *ABCB11*

Proband 3 **c.2316T**>A (p.Tyr772Stop) and **c.3589CT>GG*** (p.Leu1197Gly) in *ABCB11*

* N*ovel mutations are depicted in **bold** typeface*

Mutations in *ATP8B1*, encoding the putative aminophospholipid flippase FIC1, were identified as an underlying cause of both mild and severe low γGT hereditary cholestasis in 1998 (89). The former classification of FIC1 disease as BRIC (benign recurrent intrahepatic cholestasis) and PFIC (progressive familial intrahepatic cholestasis) referred to the different clinical courses of cholestasis. A current view characterises the FIC1 deficiency phenotype as highly variable ("clinical continuum") (43) and the prediction of phenotype severity according to the underlying defect appears to be limited (42). Despite a thorough screen, mutational analysis in a considerable number of patients with a mild clinical phenotype consistent with FIC1 disease found no predictedly pathogenic sequence changes.

Defects in another gene, *ABCB11,* encoding bile salt export pump (BSEP) were identified as a cause of progressive familial intrahepatic cholestasis type 2 (47). Despite their different aetiologies, clinical manifestation of both PFIC1 (FIC1 disease) and PFIC2 (BSEP disease) have similar clinical manifestations. Hence, we tested the hypothesis that a clinical continuum disease existed in *ABCB11*-deficient patients and screened *ATP8B1*-negative BRIC patients (P2 and P3) for mutations in *ABCB11*. We identified 4 novel *ABCB11* mutations associated with BRIC. However, publication of our data was preceded in the same year (2004) by a study describing 11 BSEP-BRIC (BRIC2) patients.

In proband 1 (P1) we identified a novel rare type of insertion-deletion (indel) mutation in exon 24 of *ATP8B1* gene: 3122delTCCTA/insACATCGATGTTGATGTTAGG in association with a known missense mutation, 1982T>C (p.Ile661Thr), in exon 17. We proposed the mechanism of indel mutation formation and discussed its impact on ATP8B1 protein folding.

At the time of indel mutation identification, only three other *ATP8B1* mutations were known to be associated with mild FIC1 disease. The prevalent mutation, c.1982T>C was found in by far most individuals with mild FIC1 disease and screening of candidate patients with such disease was focused at first on exon 17 (89-91). Our results suggested a more variable genetic background in Caucasian population, as was subsequently proved by a large study identifying 15 distinct novel mutations in BRIC1 patients, distributed throughout the coding sequence of *ATP8B1* (42).

The second mutation found in poband P1, c.1982T>C (p.Ile661Thr), is a prevalent change associated with a highly variable clinical course, but typically without permanent liver damage (91). Recently, a study expressing the p.Ile661Thr mutant in UPS-1 cells demonstrated markedly reduced protein levels (18% of wild-type) despite normal mRNA expression. The mutant protein was however capable of interacting with CDC-50A, a chaperone necessary for exit of ATP8B1 from the endoplasmic reticulum and localisation of ATP8B1 in the plasma membrane, and was able to reach the canalicular membrane of WIF-B cells (92).

The p.Ile661Thr mutation in heterozygous form together with nonsense mutations was found in progressive forms of ATP8B1/FIC1 deficiency (42). Thus the heterozygous state is associated with either episodic or chronic progressive cholestasis.

Indel in the *FIC1/ATP8B1* gene-a novel rare type of mutation associated with benign recurrent intrahepatic cholestasis.

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Hepatol Res. 2004 Sep;30(1):1-3.

Abstract

Benign recurrent intrahepatic cholestasis (BRIC) is a rare inherited liver disease characterized by recurrent attacks of severe cholestasis with no progression to end stage liver disease. Patients have jaundice, however, serum gamma-glutamyltransferase and cholesterol levels remain within the normal range during the attacks. Three mutations in the familial intrahepatic cholestasis 1 (*ATP8B1*) gene encoding a P-type ATPase have been reported so far in patients with the autosomal recessive form of BRIC. A novel rare type insertion-deletion mutation, also called indel, was found in exon 24 of *ATP8B1* in our patient together with a known missense mutation 1982T>C in exon 17. The mechanism of the indel formation is proposed and impact of the indel mutation on the function of ATP8B1 protein is discussed.

1. Introduction

Benign recurrent intrahepatic cholestasis (BRIC, OMIM 243300) is a rare inherited cholestatic liver disease characterized by recurrent attacks of severe cholestasis and no progression to end stage liver disease. Serum levels of bilirubin and bile salts are elevated but gamma-glutamyltransferase activity and cholesterol remain in the normal range during the attacks. A gene called familial intrahepatic cholestasis 1 (*FIC1/ATP8B1*, OMIM 602397) encoding a P-type ATPase is mutated in the autosomal recessive form of BRIC [1,2] (Table 1).

Table 1
Mutations in *ATP8B1/FIC1* gene associated with BRIC

Mutation	Exon no.	Consequence	Reference
1982T>C	17	I661T	[1]
2384delGAAACCGTG	20	del795GNR	[1]
?	24	Skipped exon	[2]

2. Case report

A 32-year-old Caucasian male presented with prolonged neonatal jaundice at birth and three episodes of cholestasis in early childhood. Since 1990 the patient has had one attack with typical laboratory findings annually. Bile duct obstruction was excluded during laparotomy in childhood and by repeated ultrasonography and ERCP in adulthood. No evidence for metabolic, autoimmune or infectious liver disease has ever been found. Liver biopsies performed in the last decade showed intrahepatic cholestasis with normal liver architecture.

As no simple test specific for the disease is available, a search for the molecular defect was essential to establish the diagnosis. Written informed consents were obtained from the patient and family members, genomic DNA was extracted from peripheral blood, 27 exons were PCR amplified in 24 fragments with intronic primers derived from the GenBank sequence Acc. NT011085 and the sequencing reactions were analyzed on an automated fluorescent DNA sequencer. Cycle sequencing disclosed a known missense mutation 1982T>C (I661T) in exon 17 together with a novel defect starting at the position 3122 in exon 24 of the *FIC1/ATP8B1* gene. Both defects were present at heterozygous state. Sequencing of the cloned PCR product containing exon 24 revealed an in-frame insertion-deletion mutation 3122delTCCTA/insACATCGATGTTGATGTTAGG. Both mutations were confirmed by PCR/RFLP analysis and the novel mutation was not detected in any of 100 control alleles. Five family members were found to be heterozygous for one of the mutations identified in the index patient who appeared to be a compound heterozygote. To check the potential influence of the indel on *ATP8B1* mRNA expression or splicing, RNA was isolated from cultured fibroblasts of the patient's mother heterozygous for the indel, reverse transcribed, cDNA was sequenced and submitted to restriction analysis. Both alleles were equally expressed and no alternative splicing was observed.

3. Discussion

The novel mutation 3122delTCCTA/insACATCGATGTTGATGTTAGG is a rare type of deletion/insertion mutation called indel. Indels, representing a combination of micro-deletions and micro-insertions, are formed at specific sites of the DNA sequence. Complexity analysis [3,4] used to examine the potential contribution of local DNA sequence environment to the mechanism of the indel formation revealed an inverted repeat flanking the deletion site in exon 24. This finding allowed us to propose the molecular mechanism of the mutation (Fig. 1).

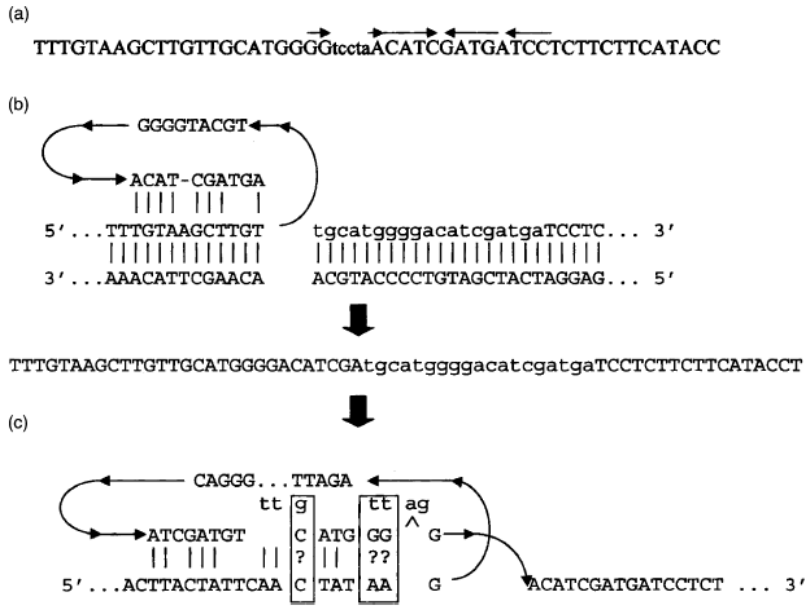

Fig. 1. Proposed mechanism of the indel formation. The first step is the deletion of TCCTA mediated by an inverted repeat (a). The deletion leads to a palindrome correction and formation of a more prominent hairpin loop structure. The second step, an insertion of a 20 bp fragment, could have occurred due to a "knot" structure formation (b). Continued DNA synthesis may then have been templated by the other strand, resulting in the duplication of the TGCATGGGGACATCGATGA sequence. The transformation of fragment CATGGGG to TTGATGTTAGG could have happened as a result of another "knot" structure formation and its subsequent correction (c).

Normal expression and splicing of *ATP8B1* mRNA isolated from mother's fibroblasts indicated the role of the indel at the protein level. Unfortunately, it was not possible to study the expression of ATP8B1 protein variant with the indel in the patient's liver directly by immunohistology because the other variant with the I661T missense mutation is known to be expressed in canalicular membrane of hepatocytes. Locations of membrane helices were calculated for the case in which the indel containing protein is expressed.

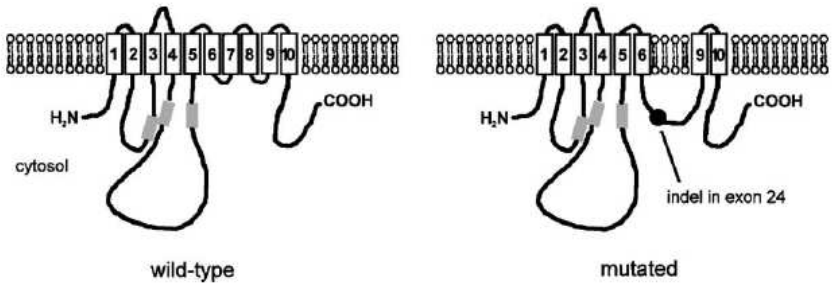

Fig. 2. Prediction of membrane domains of the wild-type and indel mutated *ATP8B1* protein. Locations of transmembrane helices were calculated by TMHMM v. 2.0 program available at the CBS server of the Center for Biological Sequence Analysis at the Technical University of Denmark. The regions proposed for the wild-type protein are in excellent agreement with the published data [1]. The disappearance of two membrane domains from the mutated protein is explained by substitution of two hydrophobic amino acids 1041Val-Leu in the middle of the seventh membrane domain by the sequence Asp-Ile-Asp-Val-Asp-Val-Arg that contains four amino acids with the charged side chain.

Regarding the predicted disappearance of two membrane domains (Fig. 2), we assume that misfolding similar to that proposed for exon 24 skipping mutation reported by van Ooteghem [2] may disturb the yet unknown function of the ATPase.

In summary, we report the case of BRIC presenting compound heterozygote carrying a novel indel mutation in *FIC1/ATP8B1* gene, predict its impact on ATP8B1 protein folding and propose the mutation mechanism.

Acknowledgements

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3.1.2 Mutational analysis of the *ABCB11* gene in Czech patients with severe phenotype (Progressive Familial Intrahepatic Cholestasis type 2 - PFIC2)

A study of two Czech paediatric PFIC patients identified the following defects:

P1: c.1445A>G (p.Asp482Gly) and **c.937C>A (p.Arg313Ser)** in *ABCB11*

P2: **c.1468A>G (p.Asn490Asp)** and **c.851T>C (p.Val284Ala)*** in *ABCB11*

None mutation was found in *ATP8B1* gene in studied subjects.

** Novel mutations are depicted in **bold** typeface*

The mutation p.Asp482Gly, identified in proband P1, is a known pathogenic defect associated in homozygous form with PFIC2 (47, 48). The mutation is located within a highly conserved nucleotide binding fold (NBF) 1 domain. Expression of BSEP varies significantly for this mutation (48).

In vitro analysis demonstrated greatly reduced levels of wild-type splicing for this mutation (only 5%) due to activation of a cryptic splice site. The levels of aberrant splice product can not be modulated by exogenous addition of splicing factor SC35 (93). The resulting predominance of immature protein can be, however, overcome by treatment with 10% glycerol at 28°C. Such treatment resulted in an increase of wild-type BSEP in CHO-K1 cells, but did not fully restore expression of mature protein.

The second mutation, p.Arg313Ser, had not been previously described. It is located within intracellular loop (IC) 2. qRT-PCR indicated no detectable *ABCB11* mRNA levels for this defect.

Consistent with the pathogenicity of both mutations found in patient P1, immunostaining demonstrated no expression of BSEP protein in patient liver.

At the age of 17 months, a hepatocellular carcinoma was detected in this patient and he underwent liver transplant 2 months later.

The development of hepatobiliary malignancy has been shown to be associated particularly with *ABCB11* mutations that result in complete absence of functional BSEP protein (48, 51, 94). p.Asp482Gly mutation appears to confer a particular risk as 16% of BSEP-deficient patients who developed hepatocellular or cholangiocellular carcinoma carried this mutation (93).

The novel mutation p.Asn490Asp, identified in proband P2, is localised within the highly conserved NBF1 and is associated with normal BSEP immunostaining. In vitro expression of p.Asn490Asp cDNA in CHO-K1 cells in a study by Byrne et al. (93) resulted in greatly reduced levels of mature protein. A taurocholate transport assay demonstrated reduction of bile salt transport to 27% of normal activity. These findings correspond with the severe course of BSEP disease in our patient, who died at the age of 11 months.

The other defect identified, p.Val284Ala, is rather enigmatic in terms of its pathogenicity. Although we did not detect this change among 300 control chromosomes, Lang et al. (95) reported its presence in 2 of 190 healthy individuals and in one patient with drug-induced cholestasis. The pathogenic missense mutation p.Val284Leu was described in the same codon (96). *In vitro* expression demonstrated no protein production for p.Val284Leu (93), whereas p.Val284Ala resulted in an increased amount of protein compared to wild-type. Similar increase in BSEP levels were reported for a prevalent SNP p.Ala444Val, which was shown to be associated with both drug-induced cholestasis and ICP (95, 97-102).

Progressive familial intrahepatic cholestasis – manifestations and diagnosis in infancy.

Kotalova R., Cebecauerova D., Knisely A.S., Hrebicek M., Jirsa M.

Čes-slov Pediat 2006; 61(4):200-206.

Summary
Progressive familial intrahepatic cholestasis (PFIC) is the common name for the group of inherited autosomal recessive cholestatic liver diseases of infancy and childhood caused by defects in genes encoding canalicular proteins involved in handling of phospholipids and bile salts. The first case in the Czech Republic of PFIC confirmed at the molecular level and resulting from defects in *ABCB11*, encoding bile salt export pump (PFIC type 2), is reported in our paper. The case is presented in the context of the differential diagnosis of neonatal cholestasis with low serum concentrations of gamma-glutamyl transpeptidase activity.

Key words
Progressive familial intrahepatic cholestasis, bile salt export pump, ursodeoxycholic acid

Introduction
Progressive familial intrahepatic cholestasis (PFIC) is the common term for the group of inherited autosomal recessive cholestatic liver diseases of infancy and childhood caused by defects in genes encoding canalicular proteins involved in handling of phospholipids and bile salts. PFIC represents one end of a spectrum whose other end is benign recurrent intrahepatic cholestasis (BRIC). On histologic study in both PFIC at presentation and BRIC during a cholestatic episode, bile ducts are not obstructed or lacking; intralobular cholestasis is the dominant finding. In PFIC, but not in BRIC, mild ductular reaction, inflammation, and various degrees of portal-tract fibrosis can be seen. Cholestasis in PFIC is mostly non-remitting and progresses gradually to biliary cirrhosis. Untreated patients die from liver failure. In BRIC morbidity from cholestasis may be severe, but scarring and liver failure do not develop.
Defects in the gene (*ABCB11*) encoding bile salt export pump (ABCB11/BSEP), an ATP-binding cassette protein functioning as a transporter, have been found to cause PFIC type 2 [1]; defects in the gene (*ABCB4*) encoding multidrug resistance protein 3 (ABCB4/MDR3), an ATP-binding cassette protein functioning as a phospholipid floppase, are responsible for PFIC type 3 [2]; and defects in the gene (*ATP8B1*) encoding familial intrahepatic cholestasis 1 (ATP8B1/FIC1), a P-type ATPase functioning as a phospholipid flippase, are the cause of PFIC type 1, known also as Byler disease in the older literature [3]. All these proteins are physiologically expressed at the canalicular membrane of hepatocytes. Absence of any of them on immunohistologic study points strongly toward the respective form of PFIC.
Elevated serum concentrations of bile salts are typical for PFIC types 1 and 2, in which bile salt secretion is decreased, whereas serum concentrations of gamma-glutamyl transpeptidase activity (GGT) are normal in these disorders. Elevation of both serum concentrations of bile salts and GGT is typical for PFIC type 3. Elevation of GGT in cholestasis is generally explained by

membrane damage of apical domains of biliary epithelia, with regurgitation of GGT-containing bile into blood. Normal activity of GGT in subjects with deficient synthesis or secretion of bile salts is a consequence of low bile salt concentration in the biliary tract. The choleretic agent ursodeoxycholic acid (UDCA) is administered to many patients with cholestasis before the nature of their cholestatic disorder is fully understood. Standard laboratory examinations, extended to include determination of total serum bile salt concentrations at least two weeks after withdrawal of ursodeoxycholic acid, can provide substantial information on the aetiology of cholestasis.
In this communication we report the first case at our clinic of PFIC with low GGT in which an aetiology was established by molecular analysis.

Patient and methods
Patient: The boy is the second child of unrelated parents. His sister, 7 years old, is well. His father is healthy; laboratory examination revealed mild elevation of serum cholesterol (5.8 - 6.1 mmol/l) and triglyceride concentrations (2.8 – 3.2 mmol/l). Serum concentrations of bilirubin, aminotransferase activities, and both alkaline phosphatase activity and GGT were normal.
The mother suffered from pruritus without jaundice during the second and the third trimester of both pregnancies.
Pruritus resolved when she gave birth; serum bile salt concentrations were not determined during either gestation.
She considers herself in good health, and has had no complaints since her second pregnancy ended. Clinical-biochemistry findings in both the patient's mother and the patient's sister are normal except for cholesterol (5.2 mmol/l in both).
Our patient was born spontaneously, at term, with birth weight 3 730 g, body length 50 cm, and excellent adaptation after birth (Apgar scores 10, 10, and 10 at 1, 5, and 10 minutes). On day 3 after birth phototherapy was started for jaundice (bilirubin, 367 μmol/l). Maternofoetal ABO incompatibility with Coombs'-test reactivity was considered the cause of hyperbilirubinemia. After 3 days of phototherapy - on day 6 – the infant was discharged home. On day 17 the total serum bilirubin concentration (TBIL) was 232 μmol/l. The serum concentration of direct-reacting bilirubin was 59 μmol/l. The serum concentration of AST activity was 1.84 μcat/l, that of ALT activity 2.94 μcat/l, that of GGT 0.51 μcat/l, and that of ALP 6.2 μcat/l. TBIL values later declined, but DBIL and AST/ALT values continued to be elevated (see Table 1). GGT values, however, remained in physiological ranges. The infant was breast fed, throve well, and had pigmented stools. Since week 7, ursodeoxycholic acid was administered. At age 3 months the infant was still fully breast fed, throve well, and had normally pigmented stools. Laboratory findings are listed in Table 1. In this state the patient was admitted to our paediatric department. Usual immunologic, metabolic, and infectious aetiologies of liver disease were repeatedly excluded. Hereditary cholestasis was suspected and a core needle biopsy of the liver was performed.
Since the age of 5 months the boy has not grown (body weight at 6.5 months 6 010 g). Psychomotor development corresponds with age. Pruritus and anorexia have escalated.
Values for bilirubin and for aminotransferase and alkaline-phosphate activities remain elevated and values for serum cholesterol, triglycerides, and albumin concentrations and for prothrombin time are within expected ranges, as are α1-foetoprotein values (Table 1). Abdominal sonography indicates diffuse liver injury without masses and a convoluted gallbladder with sludge. The infant continues to nurse and in addition is fed

rice puree, maltodextrins, casein hydrolysate, and medium chain triglycerides (Pepti Junior MCT), supplemented with fat soluble vitamins A, D, E, and K. He receives ursodeoxycholic acid and the antipruritic agent dimetindenium maleinate.
The course of clinical and laboratory findings indicates an adverse trend, prognostic of impending development of liver failure. Progressive pruritus that significantly decreases the quality of life and can speed up the timing of liver transplantation is the most unfavourable symptom.

Histology and immunohistology: The liver tissue obtained at biopsy was routinely processed from formalin into paraffin. Sections cut at 4 μM were stained using routine techniques. They also were immunostained for BSEP as described elsewhere [4]. Immunostaining for multidrug resistance protein 2 (MRP2), like BSEP an ATP-binding cassette protein functioning as a canalicular transporter, was performed for comparison purposes.

Mutation analysis: Written informed consent was signed by both parents before taking blood samples for DNA analysis of the patient, his parents, and his sister. The *ABCB11 gene* was analysed by direct sequencing of genomic DNA extracted from peripheral leucocytes. All 28 exons were amplified by PCR using the intronic primers listed in table 2. Amplified fragments were gel-purified and extracted from the gel using a QIA quick kit (Qiagen, Hilden, Germany). Sequencing was performed on the automatic fluorescence DNA sequencer AlfExpress (Amersham-Pharmacia, Uppsala, Sweden). The obtained sequence was compared with the reference sequences GenBank No. NM_003742 (mRNA) and NT_005403.14 (genomic DNA). Suspect mutations were confirmed by independent PCR-RFLP studies. The same technique was used for the family analysis and for confirmation of absence of the mutations in 100 control chromosomes.

Results

Histology & immunohistochemistry: Portal tracts were slightly expanded by fibrosis. The lobule was distorted by oedema, giant-cell change, and rosetting of hepatocytes. Centrilobular venules were unremarkable. Bile pigment was seen within hepatocytes and canalicular lumina. Kupffer cells contained bile and lipofuscin. Occasional necrotic individual hepatocytes, generally bile-stained, were found. Viral inclusions and granulomata were not identified. Small quantities of stainable iron seen in Kupffer cells and hepatocytes were considered physiologic, as were minute quantities of copper-binding protein seen in several hepatocytes. Evidence of storage disorder was not recognised. Whilst BSEP was well-expressed along canaliculi in control liver (Fig. 1a), it was not detected in the liver of our patient (Fig. 1b). MRP2 was found at canalicular margins throughout the sections of both control (not shown) and patient (Fig. 1c).

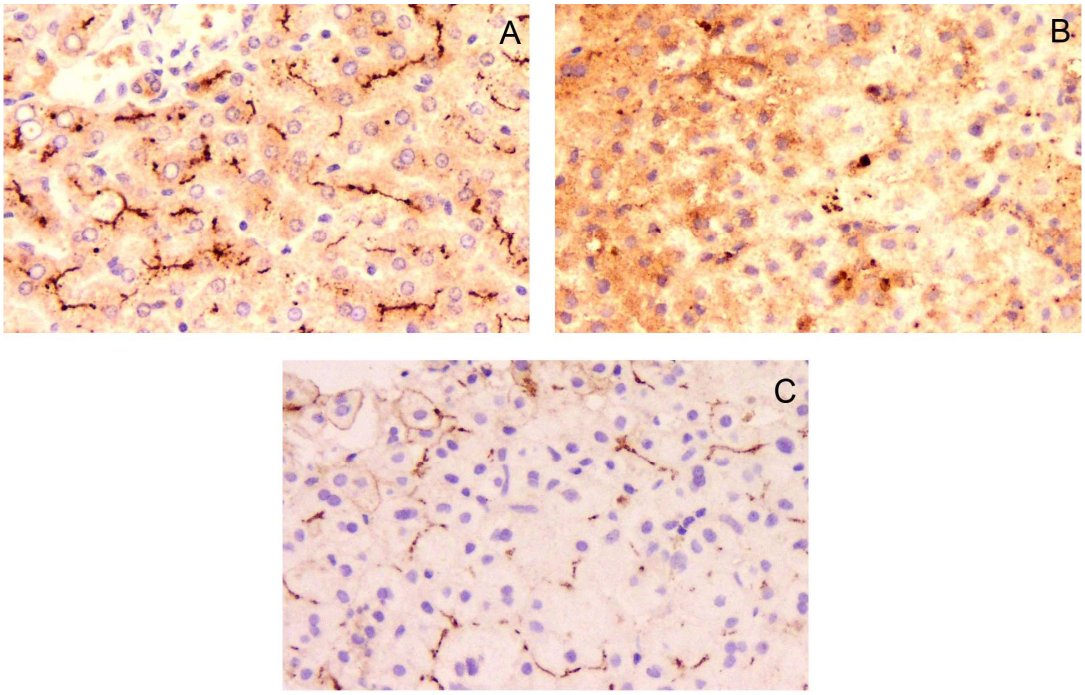

Figure 1: Immunohistology of BSEP and MRP2 proteins. In contrast to strong canalicular marking for bile salt export pump in the control liver (A), no BSEP is detectable in the liver of our patient (B). Multidrug resistance protein 2 (MRP2) is expressed both in the control (not shown) and the patient´s liver (C); slight ectopic expression of MRP2 at basolateral aspects of hepatocytes is ascribed to non-specific effects of hepatocellular injury (note hepatocyte disarray, with anisocytosis, rosetting, and multinucleation, in the patient). Polyclonal rabbit anti-BSEP antibody (gift, Y Meier / B Stieger) / hematoxylin counterstain; mouse monoclonal anti-MRP2 antibody M2II-12 (Signet / Bioquote Ltd., York, UK) / hematoxylin counterstain. Original magnification x200 all images.

Mutation analysis: Our patient is a compound heterozygote for two missense mutations in the *ABCB11* gene: a known mutation, c.1445A>G (predicted to yield Asp482Gly) [1], in protein-coding exon 13, inherited from the father (Fig. 2a), and a previously unreported mutation, c.937C>A (predicted to yield Arg313Ser) in protein-coding exon 9, inherited from the mother (Fig. 2b). The sister of our patient is heterozygous for the novel mutation c.937C>A. The HpyF3I RFLP pattern of one of 50 control subjects was identical with the pattern found in our patient, his mother, and his sister. No sequence alteration was recognized by PCR-HpyF3I RFLP in the remaining 49 control subjects.

An arginine residue at position 311, located in the second intracellular loop of the protein, is conserved in mouse, rat, and rabbit. The non-conservative substitution of Arg313Ser in a conserved sequence surrounding position 311 suggests that the maternal mutation is likely to disrupt protein function or processing. Pathogenicity of the paternal mutation c.1445A>G (Asp482Gly substitution) has been demonstrated by association of the PFIC type 2 phenotype with homozygosity for this mutation [1]. In addition to these mutations, the patient was heterozygous for two known frequent single-nucleotide polymorphisms: the non-synonymous

polymorphism c.1331T/C (Val444Ala, GenBank dbSNP rs2287622) in protein-coding exon 12 and the synonymous polymorphism c.3084A/G (Ala1028Ala, Genbank dbSNP rs497692) in protein-coding exon 23.

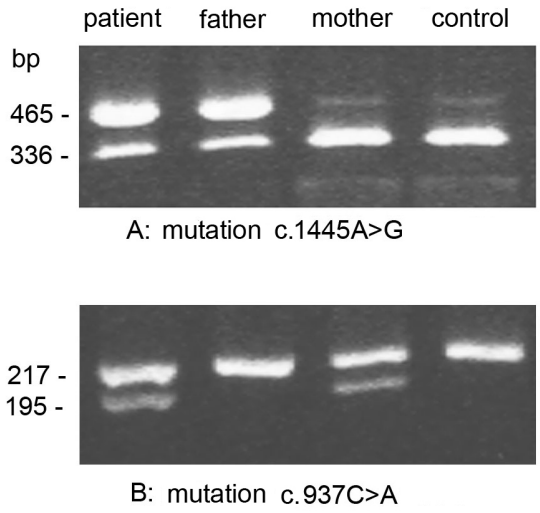

Figure 2: Segregation of the mutated alelles of the ABCB11/BSEP gene. Heterozygous mutation 1445A>G in the protein coding exon 13 was detected as PCR-FokI RFLP (A) using the sequencing primer pair No. 14 (see Tab. 1). Heterozygous mutation 937C>A in the protein coding exon 9 was detected as PCR-DdeI RFLP (B). A mutated forward primer 5´-GAGAAAAATCTTGTGTTCGCCCtG-3´ carried the single nucleotide substitution a instead of t at the second position from the 3´- end (underlined). This substitution permitted detection of mutation following the 3´- end of the primer. The reverse sequencing primer from pair No. 10 (Tab. 1) was used as the second primer.

Discussion

The incidence of PFIC in the Czech Republic is unknown. The incidence of PFIC in the United States is approximately 1/70,000 births (http://rarediseasesnetwork.epi.usf.edu/clic/).
The diagnosis will probably not be rarer in the Czech Republic. Additional cases likely are hidden under other diagnoses such as neonatal hepatitis of infants and cryptogenic cirrhosis in children.
In our patient, who represents the first case of PFIC (PFIC type 2) detected in the Czech Republic, the correct diagnosis was indicated by low GGT together with elevated levels of conjugated bilirubin and of aminotransferase activity. The diagnosis was quickly established by molecular-genetic examination. This found mutations in *ABCB11* that were predicted to lead to abnormal forms of BSEP, with decreased BSEP function. This prediction was confirmed when immunohistochemical study found that BSEP was not expressed in the patient's liver.
Intrahepatic cholestasis of pregnancy, manifest as pruritus in the patient's mother, may reflect her carrier status for a function-reducing mutation in *ABCB11*. Whether carrier status for the same mutation in the patient's sister suggests that she is at risk for cholestasis associated with

shifts in hormonal milieu (should she be advised to use barrier rather than pharmaceutical contraceptives?) is a point of interest.

The differential diagnosis of cholestasis in infancy and childhood is difficult because clinical and laboratory manifestations overlap extensively for liver injury of different aetiologies. Early establishment of the cause of cholestasis is a prerequisite for establishing an adequate approach to the individual patient. Normal or slightly elevated serum GGT activity in an infant with cholestasis is uncommon and typical for a specific group of inherited cholestatic liver diseases. In addition to PFIC types 1 and 2, disorders of bile salt synthesis [5], abnormal bile salt conjugation [6] and increased regurgitation from leaky bile ducts [6] should be considered. Severe hepatocellular insufficiency and Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome [7] can generally be excluded on clinical grounds.

Determination of total serum bile salt concentrations represents the first step towards the diagnosis provided that the patient does not receive ursodeoxycholic acid for at least two weeks. Only the value obtained under such conditions reflects the actual level of primary bile salts. Normal or low serum primary bile salts values in a patient with cholestasis are consistent with disorders of bile salt synthesis whereas elevated levels of primary bile salts are typical for the other diseases mentioned above. Recognition of defects in bile acid synthesis and conjugation is based on fast atom bombardment ionisation-mass spectrometry (FAB-MS) and gas chromatography-mass spectrometry (GC-MS) of urine and serum. The full spectrum of techniques is available in only a few centers, and was first accessible in the Czech Republic only after this child was evaluated. We believe that FAB-MS and GC-MS of urine should be conducted in patients with low-GGT cholestasis early in their clinical course, before administration of ursodeoxycholic acid confuses the clinical-biochemistry picture.

Liver biopsy can offer additional information of value. In low-GGT cholestasis, if FAB-MS and GC-MS have not identified abnormal bile acid species in urine and serum of a child without the malformations of ARC syndrome and without severe hepatocellular insufficiency, PFIC is the principal diagnostic consideration. Immunohistology and molecular-genetic examination are the tools of choice for diagnosis of PFIC. Immunohistologic evaluation is important in the diagnosis of both low-GGT and high-GGT forms of PFIC. It can direct mutational analysis toward particular genes and can confirm that identified mutations lead to abnormalities in gene-product expression. Antibodies against BSEP and MDR3 that react in formalin-fixed, paraffin-embedded tissue are available; with respect to FIC1, however, snap-frozen tissue must still be studied. Immunohistochemical studies can not, however, identify dysfunction of an expressed protein. Definitive diagnosis thus continues to require molecular-genetic analysis in the patient and the family members.

Treatment of PFIC is difficult. Depletion of the body bile salt pool may relieve symptoms of low-GGT PFIC and may slow liver scarring [8, 9]. Such depletion can be achieved by surgical interruption of the enterohepatic circulation of bile salts. In partial external biliary diversion, a loop of bowel is interposed between extrahepatic biliary tract and skin, with loss of bile salts through a cutaneous stoma [8]. In terminal ileal exclusion, bile salts are lost in the feces when chyme is prevented access to the portion of distal ileum in which most bile salt absorption occurs [9]. Experience with these techniques has yet to be acquired in the Czech Republic.

The management of PFIC is further complicated by emergence of hepatobiliary malignancy as a possible complication of PFIC [10], in particular of PFIC type 2 [11, 12]. This aspect of PFIC type 2 must be addressed in the follow-up protocol (monitoring of α1-foetoprotein levels, regular abdominal sonography evaluation). It also underscores the value of determining in low-GGT

PFIC whether PFIC type 1 or PFIC type 2 is present, as hepatobiliary malignancy has not to date been reported in PFIC type 1. Another reason to take care in classifying low-GGT PFIC is that liver transplantation seems to give better results with PFIC type 2 than with PFIC type 1. Extrahepatic features of disease in PFIC type 1, associated with expression of ATP8B1 outside the liver, may persist or become more prominent after liver transplantation. ABCB11 expression, by contrast, appears restricted to the liver, so that liver transplantation may entirely cure disease. What approach to take in our patient – diversion? List for transplantation, with symptom control rather than end-stage disease or malignancy as the principal indication? – is under active debate.

Acknowledgement
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Table 1: Laboratory findings

	Day 17	**Ref. range < 6 weeks**	**Month 3**	**Month 7**	**Ref. range 6 wks – 1 yr**
TBIL (μmol/l)	232	< 29	53	135	< 29
DBIL (μmol/l)	59		36	106	0.27 – 0.97
AST (μkat/l)	1.84	0.38 – 1.21	10.95	12.7	0.15 – 0.85
AST (μkat/l)	2.94	0.15 – 0.73	9.03	8.3	1.44 – 8.0
ALP (μkat/l)	6.2	1.2 – 6.3	12.6	7.61	1.44 – 8.0
GGT (μkat/l)	0.51	0.37 – 3.0	0.40	0.44	0.1 – 1.04
Cholesterol (mmol/l)			5.4	4.2	2.6 – 4.2
Triglycerides (mmol/l)			2.7	2.0	0.9 – 2.2
α_1-foetoprotein (μg/l)			4005	623	0 – 77
Albumin (g/l)			43	47	35 – 53
Cholinesterase (μkat/l)			114	60	87 – 140
INR			0.85	1.18	0.8 – 1.2

Table 2: Sequence of primers used for amplification of *ABCB11* gene from genomic DNA.

No.	Forward Primer	Reverse Primer	Exon
1	*5´*-ttagggacattgatccttaggc-*3´*	*5´*-cagaaacactccactccacaaag-*3´*	-1
2	*5´*-ccaaattgttctttcgtttgg-*3´*	*5´*-tgctccttgaaacttgaccag-*3´*	1
3	*5´*-aatttcttaatgactgcgttgc-*3´*	*5´*-tgaagtgcaatgtgcatgaaag-*3´*	2
4	*5´*-agccagtggggattttctttc-*3´*	*5´*-ggataggatttgtatgctttatgagc-*3´*	3
5	*5´*-cccatgaaatttggtgttgag-*3´*	*5´*-tccagctcagccagtaaaatc-*3´*	4
6	*5´*-aatccctctatatccacaaatg-*3´*	*5´*-tggcaacacattgcatctc-*3´*	5
7	*5´*-ttactttccccctttctcaac-*3´*	*5´*-aagccatgccacatatgaaag-*3´*	6
8	*5´*-tgctatccaagggtgataggg-*3´*	*5´*-caggaaaagggactcaagcttc-*3´*	7
9	*5´*-ccatgccacatgttaaatgaatc-*3´*	*5´*-ccgctttgcacaaactgag-*3´*	8
10	*5´*-ctgaagctgctctgtgtttgc-*3´*	*5´*-cctgaaggcaccaaagtaataaac-*3´*	9
11	*5´*-tctctgcgttaacatggaagac-*3´*	*5´*-ggtaaattcttcaggagttcattctg-*3´*	10
12	*5´*-acattgcagagatacgccaaag-*3´*	*5´*-aatgagcaatttgtggttatgc-*3´*	11
13	*5´*-gatttcagtggacgttgctttg-*3´*	*5´*-ctatgcatgccaggacagtctc-*3´*	12
14	*5´*-ttctgcccattggtcaagtatg-*3´*	*5´*-tcaggcatgaaactaaaacatgg-*3´*	13
15	*5´*-aagtcggagcagtgaaaattcg-*3´*	*5´*-tttcctgcagcagcacaag-*3´*	14
16	*5´*-tggattctgaaatgatgcaaag-*3´*	*5´*-ataacgcctgccagagttgttg-*3´*	15
17	*5´*-cagaatgttttggcatttgacatag-*3´*	*5´*-cagagtttccttgttgtacctgag-*3´*	16
18	*5´*-acttggacaccagttgatcctg-*3´*	*5´*-gcttaaagggtacccaacagtc-*3´*	17
19	*5´*-ctgtgaatgccaaaggatctg-*3´*	*5´*-tgtgtgatggaggcttagg-*3´*	18
20	*5´*-gatccacagcttacattaggg-*3´*	*5´*-aaacatgcaggtgattgtcag-*3´*	19+20
21	*5´*-ggtctgacctttttgtgtaatgg-*3´*	*5´*-gacagcttccttcagtctcttcg-*3´*	21
22	*5´*-ctgccatttgcaccatctaatc-*3´*	*5´*-tggtttgctaagcagcaaaaag-*3´*	22

23	*5´*-tcaaggatatttggtcctttcc-*3´*	*5´*-ccacaccatcccctgaca-*3´*	23
24	*5´*-ctttggcagcatggtttgaag-*3´*	*5´*-aacttgaagcccacttttaggg-*3´*	24
25	*5´*-accaaatgtcctgcataacacc-*3´*	*5´*-gctcaacctgtacactctggtc-*3´*	25
26	*5´*-acagaggagaccttgacatgag-*3´*	*5´*-tccacaaagtattgccaatttc-*3´*	26
27	*5´*-ttgggtttgccgtcaagtatag-*3´*	*5´*-actgagtggtggctgagctg-*3´*	27

Protein coding exons are numbered as 1 to 27, the 5´-untranslated exon is numbered as -1.

3.1.3 Genotype-phenotype correlations in children with PFIC2 from 109 families.

An extensive international study of severe forms of *ABCB11* deficiency (PFIC2) provides genotype-phenotype correlations in affected children from 109 families and assesses the effect of specific *ABCB11* genotypes (mutation type and location, degree of conservation) on BSEP immunostaining and risk of hepatobiliary malignancy.

The study includes two Czech PFIC2 patients (Patient 1 and Patient 2), previously diagnosed in the Laboratory of Experimental Hepatology, IKEM, which are discussed above (section 3.1.2)

Genotype-phenotype correlations and putative effect prediction in general may represent a useful diagnostic tool applicable in clinical practice, but as illustrated in this study, its wide use is limited since only 45% of mutations identified were unambiguously predicted to result in premature protein truncation or failure of protein production. The effect of most common missense mutations was difficult to envisage. Even a subtle change (missense mutations versus deletions, insertions or other large defects) may have a significant impact on gene/protein function, affecting gene expression and protein folding and/or trafficking.

The study of Klomp et al. (42) characterising mutations of PFIC1 (FIC1 deficient) patients demonstrated higher frequency of missense mutations in milder form of FIC1 deficiency (58% versus 38%), whereas nonsense, frameshift and deletion mutations were more frequent in progressive forms (41% versus 16%) of the disease. However, likewise in our *ABCB11* study, some *ATP8B1* mutations showed variable and hardly predictable phenotype or no clinical presentation in affected patients. Four mutations were present in both, BRIC and PFIC families, even their combination differed between BRIC and PFIC.

Mutation IVS23-3C>A, leading to an in-frame skipping of exon 24 and detected in homozygous form in 6 affected individuals from Dutch family, represented an illustrative example of a phenotypic continuum in *ATP8B1* deficiency. Though all family members initially presented with mild symptoms, in 2 individuals the disease become progressive and eventuated in an intermediate phenotype.

Our *ABCB11* study proved immunohistochemistry to be a valuable diagnostic method for *ABCB11* deficiency as 93% (82/88) of all patients from whom liver was immunostained had abnormal or absent BSEP expression. The study confirmed the earlier report of Knisely et al. (51), demonstrating higher incidence of hepatobiliary malignancy in children with *ABCB11*

deficiency. Here we show the importance of close follow-up of patients retaining their native liver. Patients carrying two protein-truncating mutations are especially at considerable risk since, in our series, 38% of them developed hepatocellular carcinoma or cholangiocarcinoma. The risk of hepatobiliary malignancy in PFIC2, frequently seen before the age of 2 years, speaks in favour of early liver transplantation, unlike in children with PFIC1, whose symptoms are not restricted only to the liver and in whom some features of disease persist and may even worsen after transplant (103).

Severe bile salt export pump deficiency: 82 different *ABCB11* mutations in 109 families.

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Abstract

Background & Aims: Patients with severe bile salt export pump (BSEP) deficiency present as infants with progressive cholestatic liver disease. We characterized mutations of *ABCB11* (encoding BSEP) in such patients and correlated genotypes with residual protein detection and risk of malignancy. *Methods:* Patients with intrahepatic cholestasis suggestive of BSEP deficiency were investigated by single-strand conformation polymorphism analysis and sequencing of *ABCB11*. Genotypes sorted by likely phenotypic severity were correlated with data on BSEP immunohistochemistry and clinical outcome. *Results:* Eighty-two different mutations (52 novel) were identified in 109 families (9 nonsense mutations, 10 small insertions and deletions, 15 splice-site changes, 3 whole-gene deletions, 45 missense changes). In 7 families, only a single heterozygous mutation was identified despite complete sequence analysis. Thirty-two percent of mutations occurred in >1 family, with E297G and/or D482G present in 58% of European families (52/89). On immunohistochemical analysis (88 patients), 93% had abnormal or absent BSEP staining. Expression varied most for E297G and D482G, with some BSEP detected in 45% of patients (19/42) with these mutations. Hepatocellular carcinoma or cholangiocarcinoma developed in 15% of patients (19/128). Two protein-truncating mutations conferred particular risk; 38% (8/21) of such patients developed malignancy versus 10% (11/107) with potentially less severe genotypes (relative risk, 3.7 [confidence limits, 1.7– 8.1; P = .003]). *Conclusions*: With this study, >100 *ABCB11* mutations are now identified. Immunohistochemically detectable BSEP is typically absent, or much reduced, in severe disease. BSEP deficiency confers risk of hepatobiliary malignancy. Close surveillance of BSEP-deficient patients retaining their native liver, particularly those carrying 2 null mutations, is essential.

Bile salt export pump (BSEP) deficiency is caused by mutations in *ABCB11*.[1,2] The severity of BSEP deficiency varies from progressive early-onset[1] to remitting and late-onset phenotypes.[3–7] Severe BSEP deficiency falls into the descriptive category of progressive familial intrahepatic cholestasis,[8–12] a heterogeneous group of autosomal recessive conditions that disrupt bile formation. BSEP deficiency is among disorders with low serum concentrations of γ-glutamyltransferase activity despite conjugated hyperbilirubinemia, as is familial intrahepatic cholestasis 1 deficiency caused by mutations in *ATP8B1*.[13] Both BSEP deficiency and familial intrahepatic cholestasis 1 deficiency exist worldwide. Their collective estimated incidence in Western Europe is 1 in 50,000 –70,000 births per year. BSEP, previously termed “sister of P-

glycoprotein,"[14] is a member of the adenosine triphosphate– binding cassette (ABC) superfamily and P-glycoprotein/multidrug resistance (MDR/ABCB) subfamily of transporters.[15,16] BSEP, expressed at the hepatocyte canalicular membrane, is the major exporter of primary bile acids.[17–19] It actively transports conjugated bile salts into biliary canaliculi against extreme concentration gradients. Liver disease in BSEP deficiency is ascribed to failed secretion and intrahepatocytic accumulation of toxic bile salts. Patients with the progressive form present as infants with high serum bile salt levels, pruritus, malabsorption, failure to thrive, jaundice, and cholestasis. They develop fibrosis and endstage liver disease before adulthood.[20–22] Partial external biliary diversion and ileal exclusion can relieve pruritus and, in some cases, slow disease progression.[23–27] However, most patients ultimately need orthotopic liver transplantation. We here present the mutations of *ABCB11* in 109 families with severe BSEP deficiency.

Materials and Methods

Families

Families with progressive familial intrahepatic cholestasis were recruited through referral to King's College London or the University of California, San Francisco. All procedures were conducted with informed consent as routine diagnostic investigations or under an institutional review board-approved protocol. Referrers supplied clinical data. No patient had elevated serum concentrations of γ-glutamyltransferase. Other causes of "neonatal hepatitis" were excluded, including primary disorders of bile acid synthesis in most cases. Families were defined as affected by "severe" progressive familial intrahepatic cholestasis and included in the study if at least one family member presented in infancy with cholestasis and low γ-glutamyltransferase that progressed, with clinical and biochemical markers of cholestasis persistently abnormal (absent surgical intervention). They were excluded if cholestasis ever resolved completely. Selection for likely BSEP deficiency was based on clinical and histologic data (specifically liver disease without the extrahepatic manifestations [pancreatitis, hearing loss, profound diarrhea] characteristic of familial intrahepatic cholestasis 1 deficiency and exhibiting giant cell hepatitis rather than bland cholestasis on histologic assessment [21]). Where possible, BSEP immunohistochemical analysis (30%)2,28,29 and/or microsatellite-based haplotype analysis[13,30,31] were used. The resultant subgroup was analyzed for *ABCB11* mutations. A total of 109 families (data and/or biopsy material available for 132 individuals) met the inclusion criteria of genetically proven severe BSEP deficiency (Supplementary Tables 1A–E; see supplementary material online at www.gastrojournal.org). Eighty-nine families were European (European, Australian, North American), and 20 were Central Asian/Arab, East Asian (Korean, Japanese, Chinese), South Asian (Indian, Pakistani), or African. BSEP immuno–staining was possible for 88 patients; clinical follow-up for malignancy was available in 128. In 7 families, clinical outcome and/or immunohistochemical results from a deceased sibling were included without mutational confirmation. In 5 families, only parental DNA was analyzed. We have previously reported single mutations in 8 families[1,2,32]; the second mutant allele is now identified in each. Clinical observations in 21 families have been reported previously.[28,29,33] These families are included to retain our population's mutation profile. Family number used throughout the text refer to the listing in Supplementary Tables 1A–E. Sequence and Genomic Structure of *ABCB11*

The 1321–amino acid BSEP protein is encoded by *ABCB11* on chromosome 2q24–31. *ABCB11* spans a 108 kilobase genomic region and is composed of 27 coding exons and one 5' untranslated exon (designated 1–28). *ABCB11* complementary DNA sequence (AF091582; AF136523; NM_003742) and genomic structure1 are available (National Center for

Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Mutation nomenclature[34] follows Human Genome Variation Society recommendations (http://www.hgvs.org/mutnomen/). Previously reported mutations have been revised accordingly. Mutation Detection Strategy Patients were initially screened by restriction endonuclease digestion for recurrent changes at mutation hot spots and, depending on ethnicity, for populationassociated changes. *ABCB11* exons subsequently underwent single-strand conformation polymorphism analysis in [44] patients followed by sequence analysis of exons with identified mobility changes. Latterly this was replaced by primary sequencing. All exons were sequenced until clearly damaging, or previously known, mutations were identified on both alleles. Samples with novel missense changes were sequenced throughout. Mutations were confirmed using freshly extracted DNA from affected individuals and parental DNA (as available). Missense changes were distinguished from polymorphisms by several criteria. First was their absence from ethnically matched control panels of at least 300 alleles (published, [35–38] in public databases [http://pharmacogenetics.ucsf.edu/ [39]], or within this study). Also considered was conservation across BSEP orthologues and MDR/ABCB homologues. Finally, predicted functional effects and differences in physical and chemical properties were assessed.

Statistics

The Statistics Calculator was used (http://www. cebm.utoronto.ca/practise/ca/statscal/). χ^2 testing assessed differences between groups. Using the same data and calculator, relative risk and 95% confidence limits were similarly calculated.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded liver, when available, were routinely stained and immunostained for BSEP using a polyclonal antibody raised in rabbit to the carboxy-terminal 21 amino acids of BSEP as previously described.[19] As a control for antigen preservation or protein expression deficiencies not specific to BSEP, parallel sections were immunostained for a structurally similar canalicular ABC transporter, multidrug resistance-associated protein 2, using a monoclonal antibody raised in mouse (Signet/Bioquote, York, England). Findings were evaluated by light microscopy as described.[28,29] For 6 families, immunohistochemical protocols used were as described.[2] BSEP immunostaining was classified as normal, not detected, or abnormal, where abnormal refers to either reduced intensity or focal absence. Immunohistochemical analysis preceded genetic analysis in 30% of cases and followed it in the remainder. Abnormality was judged by 2 or more investigators, all blinded to genetic status. DNA Extraction and Polymerase Chain Reaction Amplification DNA was extracted from blood and tissue samples using standard protocols. Polymerase chain reaction (PCR) amplification was conducted using Taq DNA polymerase (New England Biolabs, Ipswich, MA) and Roche Fast Start PCR systems (Roche Diagnostics, Basel, Switzerland). Primer details are provided in Supplementary Table 2. Microsatellite Marker Typing Microsatellite marker haplotype analysis was conducted across the *ABCB11* (2q24) and *ATP8B1* (18q21) chromosomal regions in consanguineous families, or those with ≥2 affected children, to determine which gene was likely mutated. Marker loci were selected from genetic maps[40] and developed from polymorphic repeats (Human Genome Mapping Project/Celera reference sequences; http://www.ncbi.nlm.nih.gov/). Primers were designed to allow multiplex analysis based on product size and fluorescent label (see Supplementary Tables 3A and B online at www.gastrojournal.org). Amplification products were separated on a 3100 Avant Genetic

Analyser, data were analyzed using GeneMapper software (all Applied Biosystems, Foster City, CA), and haplotypes were constructed. Families with non-Mendelian segregation of mutations were investigated for deletions of the *ABCB11* chromosomal region using microsatellite markers spanning 16.2 megabase of chromosome 2. Restriction Endonuclease Digestion Restriction endonuclease digestion was used to identify common or recurring changes rapidly and to screen ethnically matched control panels for novel changes. Enzymes were selected using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php; New England Biolabs or Roche Diagnostics). The common mutations E297G, D482G, R575X, R1153C, and R1153H abolish HphI, FokI, FokI, BsrBI, and BsrBI sites, respectively, while G982R creates an AlwNI site. PCR-amplified exon digestion products underwent 3%–5% agarose gel electrophoresis (Supplementary Table 4). Single-Strand Conformation Polymorphism Analysis Single-strand conformation polymorphism analysis was conducted using 12.5% acrylamide GeneGel Excel nondenaturing gels on a GenePhor Electrophoresis system (all Amersham Biosciences, Little Chalfont, England), initially at 5˚C and, if necessary for enhanced resolution, at 15˚C. Single-strand conformation polymorphism patterns were visualized by DNA silver staining (Amersham Biosciences). Products larger than 150 base pairs were digested before analysis (as above).

Sequencing

PCR products were purified using the High Pure PCR purification system (Roche Diagnostics) before direct sequence analysis using the version 3.1 Dye Terminator cycle sequencing kit (Applied Biosystems) and electrophoresis on a 3100 Avant Genetic Analyzer. Data were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) or SeqScape (Applied Biosystems) software.

Results

Eighty-two different *ABCB11* mutations were identified on 208 alleles in 109 families with severe BSEP deficiency (Tables 1–3, Figure 1, and Supplementary Tables 1A–E). Homozygosity, or compound heterozygosity, for *ABCB11* mutations was completely concordant with disease expression in all families genotyped. Fifty-two mutations were novel. Eighteen previously reported severe mutations were not detected.[1,2,5,44–47] The 82 mutations identified (Tables 1–3 and Figure 1) included 9 (11%; 4 novel) nonsense mutations, 10 (12%; 8 novel) small insertions and deletions, and 15 mutations (18%; 6 novel) predicted to disrupt pre–messenger RNA splicing. Sixteen (15%), 15 (14%), and 27 (25%) of the 109 families, respectively, carried at least one such change. Whole-gene deletions occurred on a single allele in 3 families. The affected individuals in families 12 and 51 appeared homozygous for a paternal mutation and the child in family 11 for a maternal mutation. Deletions confirmed and sized by haplotype analysis across the *ABCB11* chromosomal region accounted for apparent homozygosity. In family 12, the flanking markers were D2S156 and D2S326; the deletion included up to 12.5 megabase of sequence. [29] In family 51, the deletion occurred between markers D2S399 and LRP2 (encoding low-density lipoprotein–related protein 2) and included up to 2.24 megabase of sequence. Exact break points were not determined, but both lay outside the coding and promoter regions of *ABCB11*. Extended haplotype analysis was not possible in family 11. The remaining 55% of mutations were missense substitutions (Table 3 and Figure 1), which were present on at least one allele in 86 families (79%). Forty-five different mutations (32 novel) were identified. Thirty-two percent (26) of the 82 mutations occurred, or had been reported to occur, in ≥2 families, with 16% (13) in ≥3 families. Most frequent were E297G and

D482G, one or both of which were present in 58% of European families (52/89) and 15% of non-European families (3/20). E297G was detected in 34 European families (41 alleles) and on one allele in both an African-American and a South Asian family. D482G occurred in 20 European families (25 alleles) and on one allele in a Central Asian/Arab family. In all but 10 families, mutations were identified on both alleles. Of the remainder, in 2 families (107, 109), only maternal DNA was available; in one (108), available material was insufficient for complete sequence analysis;

Table 2. Splice Site Mutations in *ABCB11*

Splice site	Nucleotide change	Exon skippable in frame	Total no. of families in which identified	Families in current study	Ethnic origin	Previous reports (reference no.)
3′ Intron 5	c.390−1G>A	No	1	8	CA/AR	
5′ Intron 7	c.611+1G>A	No	2	21, 62	EU	28
5′ Intron 9	c.908+1delG	No	1	39	EU	1, 33
5′ Intron 9	c.908+1G>T	No	1	107	EU	28
5′ Intron 9	c.908+1G>A	No	1	82	CA/AR	
3′ Intron 13	c.1435−13_1435–8del	Yes	1	63	EU	28
3′ Intron 16	c.2012−8T>G	No	7	14–16, 40, 64, 65, 100	EU	28
5′ Intron 18	c.2178+1G>T	No	1	19	EU	
5′ Intron 18	c.2178+1G>A	No	4	18, 20, 41, 42	EU	4,[a] 28
5′ Intron 18	c.2178+1G>C	No	1	43	EU	
3′ Intron 18	c.2179−2A>G	Yes	1	11	EU	
5′ Intron 19	c.2343+1G>T	Yes	3	44, 66, 67	EU	29
5′ Intron 19	c.2343+2T>C	Yes	1	9	EU	28
3′ Intron 21	c.2611−2A>T	Yes	1	10	CA/AR	
5′ Intron 24	c.3213+1delG	No	2	7	EU, CA/AR	2

EU, European white; CA/AR, Central Asian/Arab.
[a]Denotes studies in which mutations are associated with mild disease.

Table 1. Nonsense Mutations, Deletions, and Insertions in *ABCB11*

Nucleotide change	Exon	Predicted effect	Mutation type	CpG site	Total no. of families in which identified	Families in current study	Ethnic origin	Previous reports (reference no.)
c.74C>A	2	p.Ser25X	Nonsense	No	1	18	EU	28
c.379delA	5	p.Thr127HisfsX6	Deletion		3	1–3	CA/AR	
c.1101_1102delAG	11	p.Val368ArgfsX27	Deletion		1	52	EU	
c.1139delT	11	p.Leu380TrpfsX18	Deletion		1	13	EU	
c.1145_1165del	11	p.Ala382_Ala388del	Deletion		2	14, 15	EU	
c.1416T>A	13	p.Tyr472X	Nonsense	No	1	4	CA/AR	28
c.1558A>T	14	p.Arg520X	Nonsense	No	2	59, 81	EU	
c.1583_1584delTA	14	p.Ile528SerfsX21	Deletion		2	53, 54	EU	
c.1723C>T	15	p.Arg575X	Nonsense	Yes	6	12, 13, 17, 19, 37	EU, EA	1, 2, 32, 45
c.1941delA	16	p.Gly648ValfsX6	Deletion		2	16, 55	EU	28
c.2316T>G	19	p.Tyr772X	Nonsense	No	1	5	EU	28
c.2787_2788insGAGAT	22	p.Lys930GlufsX79	Insertion		2	36	EU	5
c.2906_2917del	23	p.Lys969_Lys972del	Deletion		1	56	EU	
c.3268C>T	25	p.Arg1090X	Nonsense	Yes	3	17, 60	EU	2
c.3438delA	26	p.Val1147X	Deletion		1	57	EU	
c.3491delT	26	p.Val1164GlyfsX7	Deletion		1	58	EU	
c.3643C>T	27	p.Gln1215X	Nonsense	No	1	38	EA/AF	
c.3703C>T	27	p.Arg1235X	Nonsense	Yes	1	6	EU	
c.3904G>T	28	p.Glu1302X	Nonsense	No	3	20, 21, 61	EU	

EU, European white; CA/AR, Central Asian/Arab; EA, East Asian; AF, African.

Of 99 families in which complete sequence analysis was possible and mutations were identified on both alleles, patients were homozygous for a single mutation in 36% of cases (36/99) and compound heterozygotes for 2 different mutations in the remaining 64% (63/99). In 23 families homozygosity was associated with known consanguinity, while in 9 families, 2 copies of either

E297G or D482G were found. To assess effects of specific ABCB11 genotypes on expression of immunohistochemically detectable BSEP protein, families were grouped according to whether they carried 2 likely protein-truncating mutations, at least one missense mutation (E297G or D482G excluded), at least one copy of E297G, at least one copy of D482G, or only one identified mutation (Supplementary Tables 1A–E).

Immunohistochemical analysis was possible in 88 patients. All evaluated patients with 2 predicted proteintruncating mutations lacked demonstrable BSEP (Supplementary Table 1A). Variability in BSEP expression was greatest when either of the 2 common European mutations, E297G or D482G, was present on one or both alleles (Supplementary Tables 1C–E). Twenty-nine patients with at least one copy of E297G were immunostained; BSEP staining was not detected in 16 (55%), was deficient in 12 (41%), and was normal in 1. For 14 patients with at least one copy of D482G, BSEP staining was not detected in 8 (57%), was abnormal in 3 (21%), and was normal in 3 (21%). In total, 45% (19) of 42 immunostained patients with either of these mutations exhibited some BSEP expression. When all genotypes were considered, BSEP staining was absent in 72% (63/88), abnormal in 22% (19/88), and normal in 7% (6/88) of patients. Thus, in total, 93% (82/88) of all immunostained patients had abnormal or absent BSEP. Representative patterns are illustrated in Figure 2A–H. Outcome data (Supplementary Tables 1A–E) confirmed that patients with BSEP deficiency are at considerable risk for hepatobiliary malignancy. Fifteen percent of evaluable patients (19/128) developed hepatocellular carcinoma or cholangiocarcinoma. Correlation with genotype identified those with 2 protein-truncating mutations as being at particular risk. Thirty-eight percent of patients (8/21) with 2 predicted protein-truncating mutations developed malignancy versus 10% of patients (11/107) with potentially less severe defects, giving a relative risk of 3.7 (confidence limits, 1.7– 8.1; P = .003)

Discussion

In keeping with the severe phenotype for which study subjects were selected, at least 45% (37) of the 82 different mutations identified were predicted to result in premature protein truncation or failure of protein production (Tables 1 and 2). Of the 10 deletions and insertions identified, 8 resulted in a frameshift and the introduction of a premature termination codon, while the other 2, in-frame deletions of 4 and 7 amino acid residues, were likely to lead to protein misfolding and degradation.

Deletions were more frequent than insertions and typically involved the loss of 1 or 2 nucleotides. They were uniformly distributed throughout the gene (Figure 1). Most occurred within repeats or strings of nucleotides, suggesting origin by slippage or misalignment during DNA replication. The exception was exon 11, with 4 different deletions: 3 identified in the current study and one previously.[44] Of these, 3 arose between nucleotides 1100 and 1146, suggesting a deletion hot spot.

Of the 15 splice-site changes identified, all but 2 involved the invariant GT or AG dinucleotides, respectively, at positions +1/+2 of the 5’ donor and -2/-1 of the 3’ acceptor splice sites. The change c.1435-13_1435-8del is also predicted to be damaging. The remaining change, c.2012-8T>G, disrupted the pyrimidine tract of the already poorly conserved 3’ splice site of intron 16. Analysis of liver complementary DNA from a patient with this mutation (64) showed skipping of exon 17 (unpublished data, September 1999). The most common defects were missense mutations. Forty-five different substitutions were identified, with a missense mutation present on at least one allele in 79% of families (86/109). However, while insertions, deletions, and nonsense and splicing mutations are readily envisaged as damaging, the effect of a missense

substitution is more difficult to predict. Changes in amino acid size, charge, polarity, and hydrophobicity can all disrupt functional domains and protein structure or affect protein processing and trafficking. At the nucleotide level, changes within the coding region may disrupt sequences that enhance or repress pre–messenger RNA splicing.[48] Substitutions were considered detrimental based on usual criteria (see Mutation Detection Strategy). In all but one case, substitutions were not present in control panels. The exception was E297G, present on a single allele in 1 of 200 European white control chromosomes (http://pharmacogenetics.ucsf.edu)[39] in keeping with the high frequency of this allele among European BSEPdeficient patients. Degrees of conservation across orthologous and homologous proteins indicate the likely importance of a given amino acid. All altered residues were conserved or, in 2 cases, replaced only by conservative substitutions in BSEP orthologues in the mouse, rat, and skate (Table 3). Eighty-seven percent of missense residues were conserved or substituted only conservatively in homologous MDR/ABCB family members (multidrug resistance [MDR1] and multidrug resistance 3 [MDR3] proteins). At the biophysical level, 89% of missense mutations were predicted to change at least one aspect among charge, secondary or tertiary structure interactions, and hydrophobicity/polarity of BSEP (Table 3).[49] Sixty percent of mutations significantly changed amino acid residue charge, 51% size, and 69% hydrophobicity/polarity. Forty-three percent introduced or removed residues whose interactions typically determine secondary and tertiary protein structure. Of these, 22% introduced disulfide bridge–forming cysteines or α-helix-breaking prolines; the remainder introduced or removed the hydroxylated residues serine or threonine.

Table 3. Missense Mutations in *ABCB11*

Nucleotide change	Predicted effect	Exon	CpG site	Location	Change in:			
					Size	Charge	Hyd/Pol	Shape
c.149T>C	p.Leu50Ser	4	No	NH2 term	Y		Y	Y
c.470A>G	p.Tyr157Cys	6	No	TM2	Y		Y	Y
c.725C>T	p.Thr242Ile	8	No	TM4	Y		Y	
c.890A>G	p.Glu297Gly	9	No	IC2	Y	Y	Y	
c.908G>A	p.Arg303Lys	9	No	IC2				
c.937C>A	p.Arg313Ser	10	Yes	IC2	Y	Y	Y	Y
c.980G>A	p.Gly327Glu	10	No	TM5	Y	Y	Y	
c.1168G>C	p.Ala390Pro	11	No	TM/NBF				Y
c.1229G>A	p.Gly410Asp	12	No	TM/NBF		Y	Y	
c.1238T>G	p.Leu413Trp	12	No	TM/NBF				
c.1388C>T	p.Thr463Ile	13	No	Adj Walker A	Y		Y	Y
c.1396C>A	p.Gln466Lys	13	No	Adj Walker A		Y		
c.1409G>A	p.Arg470Gln	13	Yes	Adj Walker A		Y		
c.1415A>G	p.Tyr472Cys	13	No	Adj Walker A	Y		Y	Y
c.1442T>A	p.Val481Glu	14	No	NBF1	Y	Y	Y	
c.1445A>G	p.Asp482Gly	14	No	NBF1		Y	Y	
c.1460G>C	p.Arg487Pro	14	Yes	NBF1	Y	Y	Y	Y
c.1468A>G	p.Asn490Asp	14	No	NBF1		Y		
c.1535T>C	p.Ile512Thr	14	No	NBF1	Y		Y	Y
c.1544A>C	p.Asn515Thr	14	No	NBF1			Y	Y
c.1550G>A	p.Arg517His	14	Yes	NBF1		Y	Y	
c.1621A>C	p.Ile541Leu	14	No	NBF1				
c.1622T>C	p.Ile541Thr	14	No	NBF1	Y		Y	Y
c.1643T>A	p.Phe548Tyr	15	No	Adj ABC				
c.1685G>A	p.Gly562Asp	15	No	ABC		Y	Y	
c.1708G>A	p.Ala570Thr	15	Yes	ABC/Walker B				Y
c.1763C>T	p.Ala588Val	15	No	Adj Walker B			Y	
c.2272G>C	p.Gly758Arg	19	No	NBF/TM	Y	Y	Y	
c.2296G>A	p.Gly766Arg	19	Yes	TM7	Y	Y	Y	
c.2494C>T	p.Arg832Cys	21	Yes	IC3	Y	Y	Y	Y
c.2576C>G	p.Thr859Arg	21	No	IC3	Y	Y	Y	Y
c.2842C>T	p.Arg948Cys	23	Yes	IC4	Y	Y	Y	Y
c.2935A>G	p.Asn979Asp	23	No	TM11		Y		
c.2944G>A	p.Gly982Arg	23	Yes	TM11	Y	Y	Y	
c.3086C>A	p.Thr1029Lys	24	No	TM12	Y	Y	Y	Y
c.3329C>A	p.Ala1110Glu	25	Yes	Adj Walker A	Y	Y	Y	
c.3382C>T	p.Arg1128Cys	25	Yes	Adj Walker A	Y	Y	Y	Y
c.3457C>T	p.Arg1153Cys	26	Yes	NBF2	Y	Y	Y	Y
c.3458G>A	p.Arg1153His	26	Yes	NBF2		Y	Y	
c.3460T>C	p.Ser1154Pro	26	No	NBF2				Y
c.3628A>C	p.Thr1210Pro	27	No	Adj ABC				Y
c.3691C>T	p.Arg1231Trp	27	Yes	ABC/Walker B		Y	Y	
c.3692G>A	p.Arg1231Gln	27	Yes	ABC/Walker B		Y		
c.3724C>A	p.Leu1242Ile	27	No	Walker B				
c.3892G>A	p.Gly1298Arg	28	No	NBF2	Y	Y	Y	

NOTE. Unless specified, all proteins are human.

EU, European white; CA/AR, Central Asian/Arab; EA, East Asian; SA, South Asian; AF, African; m/r/s bsep, mouse/rat/skate bsep; IC, intracellular loop; Hyd/Pol, hydrophobicity or polarity; NBF, nucleotide binding fold; TM, transmembrane; NBF/TM, location between NBF domain and TM region; NH2 Term, amino terminal; MDR1/3, multidrug resistance proteins 1/3.

[a]Denotes studies in which mutations are associated with mild disease.

Table 3. Continued

Conservation						Total no. of families in which identified	Families in current study	Ethnic origin	Previous reports (reference no.)
BSEP	mbsep	rbsep	sbsep	MDR1	MDR3				
L	L	L	L	M	L	1	47	EU	
Y	Y	Y	Y	Y	Y	1	49	EU	
T	T	T	T	T	T	1	41	EU	
E	E	E	E	Q	Q	67	51–80, 93, 94, 102, 103, 108, 109	EU	1, 2, 4,[a] 5,[a] 29, 33
R	R	R	R	R	N	1	22	CA/AR	
R	R	R	R	R	E	1	83	EU	
G	G	G	G	G	G	1	50	EU	
A	A	A	A	A	A	1	68	EU	
G	G	G	G	G	G	1	84	EU	
L	L	L	L	P	P	1	45	EU	
T	T	T	T	T	T	1	38	EA, AF	
Q	Q	Q	E	Q	Q	1	101	CA/AR	
R	R	R	R	R	R	2	23, 85	EU, CA/AR	
Y	Y	Y	Y	Y	Y	1	46	EU	
V	L	L	L	V	I	1	36	EU	
D	D	D	D	D	D	22	81–99, 104, 105	EU, CA/AR	1, 2, 33
R	R	R	R	R	R	1	24	EU	
N	N	N	N	N	N	1	106	EU	
I	I	I	I	I	I	1	46	EU	
N	N	N	N	N	N	1	86	EU	33
R	R	R	R	R	R	1	87	EU	33
I	I	I	I	I	I	3	25, 39	EU	33, 49
I	I	I	I	I	I	1	44	EU	
F	F	F	F	F	F	1	69	EU	33
G	G	G	G	G	G	1	88	EU	
A	A	A	A	A	A	1	26	EU	4[a]
A	A	A	A	A	A	2	70, 89	EU	33
G	G	G	G	G	G	1	27	CA/AR	
G	G	G	G	G	G	1	28	CA/AR	
R	R	R	R	R	R	2	29, 90	CA/AR, EU	33
T	T	T	T	T	T	1	43	EU	
R	R	R	R	R	R	2	42, 71	EU	
N	N	N	N	H	H	1	30	EU	
G	G	G	G	G	G	4	31, 37	EU, CA/AR	1, 2
T	T	T	T	M	V	1	91	EU	
A	A	A	A	A	A	2	47, 72	EU	
R	R	R	R	R	R	1	32	SA	
R	R	R	R	R	R	4	73	CA/AR, EU	1, 47
R	R	R	R	R	R	4	33, 34, 40, 48	EU, SA	
S	S	S	S	A	A	1	50	EU	
T	T	T	T	T	T	1	35	EU	
R	R	R	R	R	R	1	92	EU	28
R	R	R	R	R	R	2	48	EU	48
L	L	L	L	L	L	1	45	EU	
G	G	G	G	G	G	1	49	EU	

While protein-truncating mutations were distributed uniformly throughout the protein (Figure 1), 60% of missense changes clustered in the 2 highly conserved nucleotide-binding fold (NBF) domains (residues 414–610, 1072–1321[50]; 38% in NBF1 and 22% in NBF2). Fourteen changes (31%) occurred within, or immediately adjacent to, the Walker motifs. Of the remainder, 7 (16%) occurred in the transmembrane (TM) domains; 5 of these introduced a charged residue into a hydrophobic domain and, in 3 cases, simultaneously removed glycines, which stabilize α-helical TM spans. The intracellular loops contained 6 changes (13%), of which 3, including

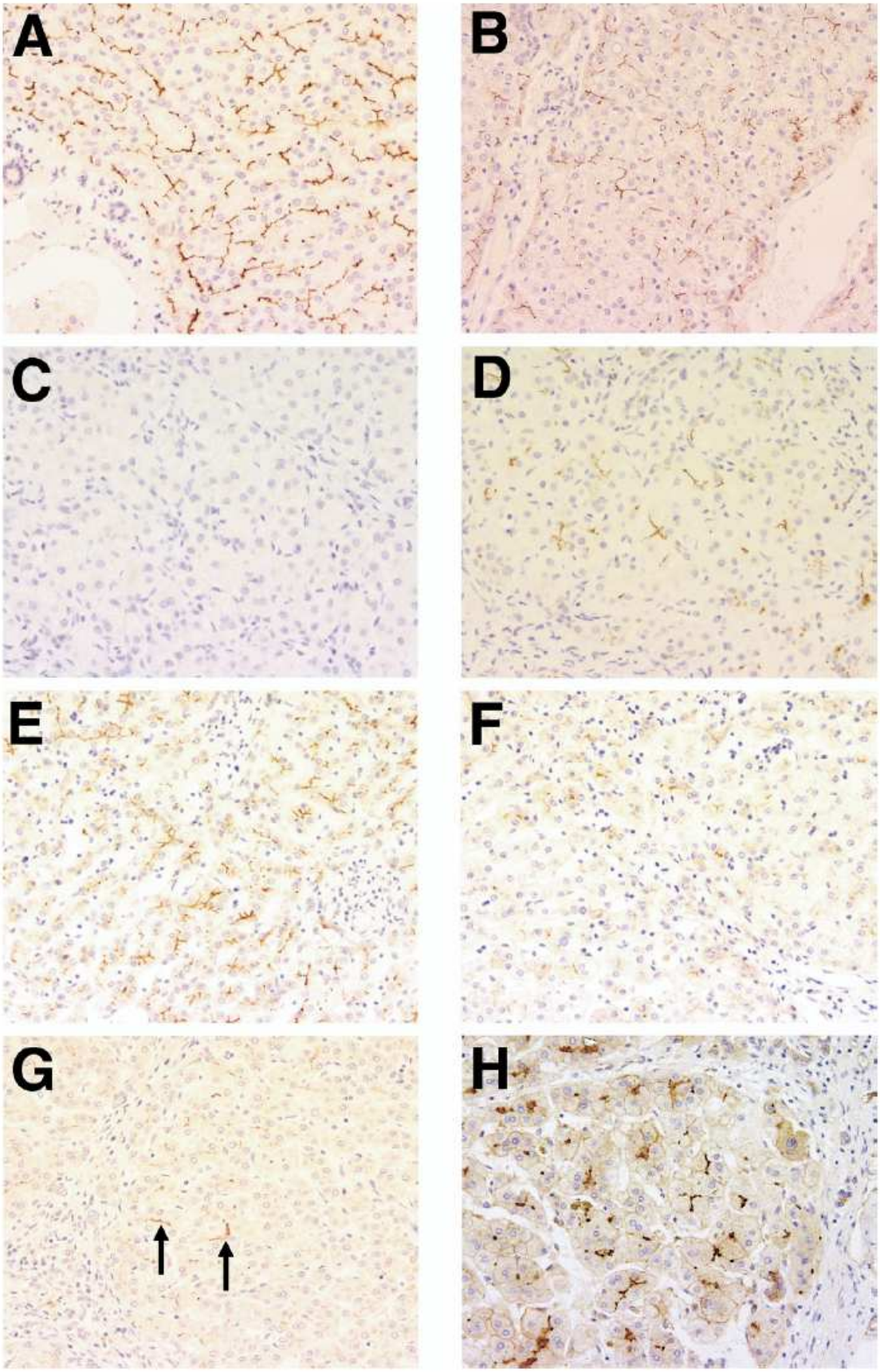
E297G, occurred in intracellular loop 2. No changes were identified in the extracellular loops, although 2 have been reported.[2,44]

In the 93% of families (99/106) in which complete sequence analysis was possible, 2 mutant *ABCB11* alleles were identified. In the remaining 7 families, only a single mutation was identified despite extensive analysis. In 5 cases, the single mutations were splice-site changes or E297G/D482G. In only 2 families were novel missense mutations identified (Q466K, N490D). Consistent with pathogenicity, both were at conserved sites in homologues and were absent from control populations. Q466K was associated with abnormal BSEP staining (detrimental). N490D was associated with normal staining; however, this mutation lies within NBF1, a region in which disease-associated mutations coexisted with retained BSEP staining (see following text). While we cannot exclude mutations in genes other than *ABCB11* in this patient, analysis of *ATP8B1* has identified no defects (unpublished data, July 2007). Most patients with cystic fibrosis in whom sequencing detects only one mutation harbor microdeletions of one or more exons.[51] This is likely also true for *ABCB11*. Such deletions, unless homozygous, will not be detected by genomic sequencing; exon-dosage analysis such as multiplex ligation-dependent probe amplification must be used. Among the 82 different mutations identified in this study, 32% (26) occurred, or had been reported, in multiple families, with 16% (13) present in ≥3 families. These likely represent both recurrent and founder mutations. The most common natural mutation hot spots are cytosine guanine (CpG) dinucleotides.[52] Thirty-three percent (18) of 54 missense and nonsense mutations occurred at these sites (Tables 1 and 3). Ten mutations occurred in multiple families: R470Q, R832C,[33] R948C, A1110E, and R1231Q[53] have now been reported in 2 families; R1090X2 in 3 families; G982R,1,2 R1153C,[1,47] and R1153H in 4 families; and R575X in 6 families.[1,2,32,45]

Six common missense and nonsense changes occurred at non-CpG sites: R520X and A588V33 in 2 European families and E1302X and I541L33,[54] in 3 European families each. By far most common, however, were E297G and D482G, at least one of which was present in 58% (52/89) of European and in 15% (3/20) of non-European families. The population distribution of E297G, most frequent along the North European seaboard, suggests an origin in Northern Europe and spread southward through Central and Eastern Europe. The mutation was also found in one South Asian and one African-American family. In contrast, D482G likely originated in Central or Eastern Europe, with cases identified in Polish, Austrian, Slovak, Czech, Hungarian, and Russian families. This mutation was also present in a Greek and an Iranian family. Five common insertions and deletions were identified, with c.379delA in 3 apparently unrelated Arab families and c.1145-1165del, c.1583_1584delTA, c.1941delA,[28] and c.2787_2788insGAGAT[5] in 2 European families each. Five common splice-site mutations occurred, with c.611+1G>A,[28] c.2012-8T>G,[28] c.2178+1G>A,[4,28] c.2343+1G>T,[29] and c.32131+1delG2 in 2, 7, 4, 3, and 2 families, respectively. Geographic distributions suggest that most are likely founder mutations. Mutation clusters were also observed. For NBF domains, this likely reflects functional importance. At other sites, both mutations and polymorphisms clustered within the same or adjacent codons, suggesting sequence instability or mutagen interaction. Mutation clusters include Y472C, Y472X,[28] and I541L[33,54]/I541T. Four different changes occurred at, or adjacent to, the 5' splice site of intron 9: R303K, c.908_1delG,[1,33] c.908_1G_T,[28] and c.908_1G_A. Three occurred at the 5' splice site of intron 18: c.2178_1G_A,[4,28] c.2178_1G_T, and c.2178_1G_C. BSEP deficiency represents a phenotypic continuum between progressive early-onset1 disease and remitting, and late-onset, phenotypes.[3–7] Eleven different mutations have been reported in BSEP deficiency disease clinically assessed as intermediate[6] or mild in severity.[4,5,7] Three of

these, E297G, A570T, and c.2178_1G_A, have also been found in progressive familial intrahepatic cholestasis. In milder disease, missense mutations predominate over those that result in protein truncation or a failure of protein production, more mutations occur in less conserved regions (eg, intracellular loops) than in the NBFs, and mutated residues are less likely to be conserved. Compound heterozygosity for both a severe and a mild mutation may result in mild disease. That homozygosity for E297G is seen in discrepant phenotypes strongly indicates that environment and genetic background also play roles.

Immunohistochemistry appears valuable in identifying likely BSEP deficiency, because many patients studied exhibited little or no detectable BSEP at the hepatocyte canaliculus.[2,28,29] An anti-C-terminal antibody can be expected not to mark when protein is absent or when a misfolded or truncated "partial protein" is expressed. Immunoreactivity may depend on tissue processing, disease stage, therapeutic intervention, and disease mechanism. These factors are to be considered in interpreting immunohistochemical results. Immunohistochemically detectable BSEP expression does not exclude functional BSEP deficiency. Twenty-eight percent of patients (25/88) analyzed exhibited some degree of BSEP staining; in 6, expression was considered normal. Residual staining was most striking in patients with E297G or D482G, with some seen in 45% of patients (19/42) carrying at least one of these alleles. Ten additional missense mutations were associated with detectable BSEP staining (Supplementary Tables 1*B*–*E* and Figures 2*A*–*H*). Abnormal BSEP staining was seen with L50S, Q466K, N515T, R517H, I541L, and F548Y and normal with N490D, G562D, R832C, and A1110E. Because most of these were found in combination with E297G or D482G, their individual effects could not be assessed. Nine of the 12 mutations occurred within the highly conserved NBFs (8 within NBF1, 4 within or adjacent to Walker motifs), suggesting an effect on protein function.


A
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Abnormal (typically reduced) staining may also result from decreased protein production or defective sorting or instability of an otherwise functional protein. Such patients in particular may be amenable to therapeutic interventions such as partial external biliary diversion or the use of pharmacological agents that enhance BSEP cell surface expression (eg, 4-phenylbutyrate).[55]
The above notwithstanding, not to detect BSEP immunohistochemically, or to show BSEP deficiency, is highly useful. Deficiency or absence of BSEP expression could be shown in 93% (82) of the 88 patients in whom immunohistochemical study was possible. As some patients were selected for mutational analysis in part because they lacked immunohistochemically detectable BSEP, the exact sensitivity of this technique could not be assessed. We have previously shown that BSEP deficiency is associated with increased susceptibility to hepatobiliary malignancy.[28,29] In this study, 38% of patients (8/21) with 2 predicted protein-truncating mutations (Supplementary Table 1A) developed either hepatocellular carcinoma or cholangiocarcinoma versus 10% (11/107) of other patients (Supplementary Tables 1B–E), giving a relative risk of 3.7 (confidence limits, 1.7–8.1; P = .003). Of the other 13 patients with 2 protein-truncating mutations, all but 3 have undergone orthotopic liver transplantation or died of non-malignant disease. The 3 who retain their native livers (ages 2.4, 7, and 16 years) are under close observation. Without orthotopic liver transplantation, the incidence of malignancy in patients with 2 protein-truncating *ABCB11* mutations is expected to exceed that observed in this study. BSEP-deficient patients, in particular those carrying 2 predicted null mutations, who retain their native liver require close surveillance for hepatobiliary malignancy.

Supplementary Data Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.01.038.

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3.1.4 Characterisation of the regulatory regions of the *ATP8B1* gene

Despite the fact that the *ATP8B1* gene was identified in 1998 (89) as a cause of both progressive and mild intermittent forms of intrahepatic cholestasis in 1998, only the coding region of the gene had been characterised. The identification of the non-coding parts of *ATP8B1* is an essential prerequisite for further studies of PFIC1 patients. The 5'UTR and promoter region(s) represent a promising target for mutational search in patients whose clinical disorder is consistent with FIC1 deficiency or in whom genetic findings are consistent with linkage to *ATP8B1* and in whom no mutations within protein-coding parts of the gene are found. Mutations in the regulatory regions may represent a significant portion of such patients, especially those with milder phenotypes.

In a large study by Klomp et al. (42) *ATP8B1* mutations were detected in fewer than 50% of the patients screened, irrespective of severity of their disease. A similar study of 109 PFIC2 families identified only a single heterozygous mutation in *ABCB11* in 7 families, despite extensive sequence analysis (48).

The high number of patients in whom no pathogenic mutations or only single heterozygous mutations were identified highlights the limits of conventional sequencing approach. Some larger defects such as exonic deletions are not detectable, unless homozygous, by convential sequencing and require methods based on exon-dosage analysis such as multiplex ligation-dependent probe amplification (MLPA). Mutations within introns, especially those located outside exon-adjacent regions, represent another uneasy goal for mutational detection strategies. Some mutations may involve yet unidentified regions such as 5' and 3' UTRs or upstream regulatory parts of the gene.

PFIC and BRIC types 1 and 2 manifest with a largely shared phenotype irrespective of the underlying defects. Overlap in clinical presentation also can be seen in familial hypercholanemia and defects of BA biosynthetic pathway. Thus the genes involved in these disorders might be feasible candidates for mutational search in patients without demonstrable *ATP8B1* mutations. In addition, that the another locus for low γGT cholestasis exists, was suggested (104).

Thus the application of new techniques, analysis of as yet unidentified regulatory regions of *ATP8B1* and *ABCB11* genes and search for additional disease loci in patients with no mutation

found or only a single mutation found represents the current challenge in the field of low γGT cholestases.

We have contributed to meeting this goal by identification of 5'UTR and promoter regions of the *ATP8B1* gene.

Our work has demonstrated the complex structure of the *ATP8B1* gene, identifying novel untranslated exons and three independent promoter regions. In liver, the promoters P1 and P2 play only minor role under the physiological conditions. Promoter P3 is located within a CpG island and was proved to be the essential regulatory element responsible for 70% of total gene expression. This region displays the typical features for promoters of housekeeping genes: It consists of TATA-less, GC-rich sequence with multiple transcription start sites. In addition, only non-specific putative transcription factor binding sites, Sp1, AP-2 and NFκB, were identified within the proximal promoter P3. We further demonstrated no significant link between bile acids - farnesoid X receptor and the main promoter of *ATP8B1*. The *ATP8B1* promoter's characteristics are in agreement with ubiquitous expression of FIC1 protein, including both organs involved in bile acid circulation and those apparently unrelated to bile acid homeostasis such as lung, heart, placenta and stereocilia of inner ear (89, 105, 106). Our results thus support uniform, bile acid-independent mechanisms regulating *ATP8B1* basal expression and function across different tissues.

ATP8B1 gene basal expression is driven by a housekeeping-like promoter independent of bile acids and FXR.

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To be submitted.

Abstract

Background: Mutations in the *ATP8B1* gene were identified as a cause of low γ-glutamyltranspeptidase cholestasis with variable phenotype, ranging from Progressive Familial Intrahepatic Cholestasis to Benign Recurrent Intrahepatic Cholestasis. *ATP8B1* was mapped to chromosome 18, however only the coding region of the gene has been described. The aim was to analyze the regulatory regions, promoter and 5'untranslated region, of the *ATP8B1* gene.

Methods: 5'Rapid Amplification of cDNA Ends (5'RACE) using liver and intestinal tissue was performed to search for the presence of 5' untranslated exons. Expression levels of *ATP8B1* transcripts were determined by quantitative reverse-transcription PCR (qRT-PCR) and compared with non-variable part of *ATP8B1*. Three putative promoters were examined *in vitro* using reporter gene assay and the main promoter was stimulated with chenodeoxycholate acid (CDCA).

Results: Four novel untranslated exons located up to 71kb upstream of previously published exon 1 and ten different splicing variants were found both in liver and intestine. The multiple transcription start sites were identified within exon -3 and proximal promoter upstream of exon -3 was proved to be an essential regulatory element responsible for 70% of total gene expression. *In vitro* analysis demonstrated that main promoter drives constitutive *ATP8B1* gene expression independent of bile acids.

Conclusion: The structure of the *ATP8B1* gene is complex and the previously published transcription start site is not significant. The basal expression of *ATP8B1* is driven by the house-keeping like promoter located 71kB upstream of the first protein coding exon.

Introductory statement

Mutations in the *ATP8B1* gene cause low γ-glutamyltranspeptidase (γ-GT) cholestasis with a variable phenotype ranging from progressive to benign recurrent forms (Progressive Familial Intrahepatic Cholestasis type 1, formerly Byler disease, and Benign Recurrent Intrahepatic Cholestasis type 1, BRIC1; OMIM 211600, 243300).

ATP8B1 deficient patients suffer from intrahepatic cholestasis, elevated serum bile salts and aminotransferases along with normal γ-GT and cholesterol. They often develop extrahepatic symptoms including diarrhoea, pancreatitis and hearing problems. Milder phenotype presents with recurrent attacks of cholestasis without permanent liver damage.

The exact mechanism leading to cholestasis is poorly understood. The ATP8B1 or FIC (Familial Intrahepatic Cholestasis 1) protein, a member of P4-type ATPases subfamily, is expressed in the apical membrane of many tissues including enterocytes, pancreas and to a lesser extent hepatocytes and cholangiocytes. On the basis of its homology with yeast aminophospholipid flippases and studies showing increased translocation of phosphatidylserine (PS) in cells

transiently or stably expressing ATP8B1 (1, 2) the protein is considered a phosphatidylserine flippase, translocating PS from the outer to the inner leaflet of the plasma membrane.
In vivo experiments using "Byler" Atp8b1$^{G308V/\ G308V}$ mice or ATP8B1 deficient human and rat hepatocytes demonstrated defective membrane order due to the impaired flippase activity of ATP8B1 (3, 4). Consequent loss of membrane asymmetry resulted in an impaired resistance of canalicular membrane to hydrophobic bile salts and the changed cholesterol to phospholipids ratio was shown to affect the activity of main canalicular bile salt transporter BSEP (Bile Salt Export Pump) (5). Disrupted order of the plasma membrane and its higher sensitivity to a detergent effect of bile salts is well-illustrated by several findings typical for patients suffering from PFIC 1 but not PFIC 2 (ABCB11/BSEP deficiency) or other intrahepatic cholestases: i) coarsely granular 'Byler bile' that is proposed to be a mixture of cholesterol and membrane remnants, ii) loss of microvilli, and iii) increased extraction of ectoenzymes from canalicular membrane (3, 6). More recent study, however, challenged the proposed mechanism of a lost membrane asymmetry as a consequence of impaired aminophospholipid transport (7) and proposed a dual role for ATP8B1. Whereas flippase activity was not affected in ATP8B1-depleted CaCo-2 cells, impaired function of ATP8B1 resulted in perturbation of microvilli formation.
Some investigators attempt to attribute the cholestasis in PFIC1 patients to the disruption of FXR-mediated regulatory pathway, proposing the enhanced ileal absorption of bile acids as a putative contribution mechanism leading to cholestasis. (8). As the reduced hepatic expression of FXR has been demonstrated in patients with other cholestatic diseases, decreased FXR levels are apparently secondary due to cholestasis (9).
Recently, controversial data were published about the possible relationship between *ATP8B1* and FXR nuclear receptor (4, 8-12). Experiments with heterogously expressed ATP8B1 in UPS cell model demonstrated 6-fold increase in BSEP promoter activity, the phenomenon which was lost after mutation of FXR response element in the promoter of *ABCB11* gene (11). On the basis of hereby induced/lost *ABCB11* promoter activity, ATP8B1→FXR→ABCB11 regulatory pathway was suggested. On the contrary, experiments using small interfering RNA (siRNA) on human and rat hepatocytes and CaCo-2 cell line did not show any change at mRNA and protein levels of FXR and ABCB11 (BSEP) in ATP8B1/atp8b1-deficient cells. (4) Similarly, unperturbed activity of FXR and its target genes was observed in ATP8B1-depleted Caco-2 cells using small hairpin RNA (shRNA), targeted against the coding region of *ATP8B1* (van der Velden et al., manuscript in preparation). These results suggest an unimpaired FXR signalling pathway in PFIC1 patient.
Little is known about the regulation of the *ATP8B1* gene expression, although the protein coding part of the *ATP8B1* gene comprising 27 exons is well characterised. Our aim was to characterise transcriptional regulation of the *ATP8B1* gene by identifying its promoter and 5´untranslated (5'UTR) region and search for putative regulatory sites in newly discovered parts of the gene.

Material and methods

5' Rapid amplification of cDNA ends (5'RACE). The 5´ends of the *ATP8B1* gene were mapped using RACE technique (5'/3' RACE kit, 2nd Generation, Roche, Switzerland) according to manufacturer instructions. Total RNA was isolated using RNA-Bee (Tel-test, Inc.) and 50mg liver or intestinal tissue, or 5x10^6 of HepG2 cells. Tissue was obtained from donors after previous written informed consent. To confirm newly identified transcriptional origins, liver and intestinal RACE ready cDNA (Ambion, Austin, USA) was used. The *ATP8B1 g*ene specific primers for reverse transcription were localised in the exon 3-4, 2-3 and 1-2 boundaries,

respectively. PCR products were then cloned into the pDrive Cloning Vector (Qiagen, Hilden, Germany) and sequenced using ABI Big Dye (Version 3.1) on 3100 automated DNA Sequencer (Applied Biosystems, Foster City, USA) in both directions using vector specific primers.

Quantitative Real–time PCR (qRT-PCR). Nine sets of individually designed TaqMan® MGB probes and primers were generated using Primer Express® Software Version 2.0 (Applied Biosytems, Warrington, UK), to cover all variants of alternative splicing of the untranslated exons. Supplement Fig 2. Probe designed to a non-variable translated exonic region served as a calibrator of the transcript levels. All probe sets were designed across exon-exon boundaries to eliminate the possibility of genomic contamination.
The amplification efficiency for each probe was tested on control templates obtained by cloning of the appropriate cDNA region using different concentrations of both, positive and negative controls. As each probe set worked with different efficiency**,** the concentration of probes was adjusted for each positive control to reach a cycle threshold (Ct) value difference no greater than 1.
100 ng of DNase-treated total RNA from normal human liver and intestinal samples was used as a template in a 20 µl reverse transcription reaction using Transcriptor (Roche, West Sussex, UK) of random hexamer or gene specific primers (Invitrogen, Paisley, UK, Sigma-Aldrich, Dorset, UK). 1 µl of single strand cDNA was then assessed in triplicate for levels of the different *ATP8B1* transcripts on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Levels of the untranslated transcripts and the non-variable calibrator of the *ATP8B1* gene were corrected to the level of 18S rRNA. A PCR of non-reverse transcribed RNA was performed as a negative control to check for any genomic DNA contamination. Delta Ct (dCt) values were calculated using ABI SDS software with RQ study application (Version 1.2.3, Applied Biosystems) and the data analysed using Excel.

Promoter and first exon analysis *in silico*.
Three independent algorithms for promoter prediction (http://genome.ucsc.edu/, http://www.genomatix.de, http://bimas.dcrt.nih.gov/molbio/proscan/) were used to analyse the 5'UTR of the *ATP8B1* gene. The University of California Santa Cruz Genome Bioinformatics server was also used to predict the gene's first exon. The data obtained were compared with EST database and the 5' RACE experimental results.

Plasmid construction. Twelve fragments of the 5´UTR (Fig.5) of the *ATP8B1* gene were PCR amplified using human genomic DNA as a template, *Pfx* polymerase (Invitrogene) and specific primers with XhoI restriction sites. PCR product was cloned (Invitrogen Zero blunt kit or Qiagen Cloning kit), sequenced, digested with XhoI and ligated into XhoI predigested luciferase reporter gene vector pGL3-Basic (Promega, Southampton, UK) using Quick Ligation Kit (New England Biolabs, Hitchin, UK). Resulted constructs were isolated with Endo Free Maxi kit (Qiagen, West Sussex, UK) and checked for the correct sequence with various restriction enzymes and direct sequencing.
pCI human retionid X receptor α (pCI_hRXR α) and pCI human farnesoid X receptor (pCI_hFXR) were obtained from Jane Byrne.

Cell culture and transfection. HepG2 cell line was purchased from ATCC. Cells were maintained in Dulbecco´s modified Eagle´s medium (DMEM, PAA, Farnbourough, UK)

supplemented with 5% or 10% fetal calf serum (FCS, PAA). rNTCP-HepG2 cells, kindly provided by Ulrich Beuers (Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands) and Christopher Rust (Department of Medicine 2 – Grosshadern, University of Munich, Munich, Germany), were maintained in DMEM containing 5% FCS and 1mg/ml G418 (Invitrogen). Cells were regularly tested for *Mycoplasma* contamination. For transfection, cells were seeded in 24-well plates (TPP) in medium containing 5% FCS or 5% charcoal-stripped bovine calf serum (GibcoBRL). For transient transfection 1.5µl of FuGene HD (Roche) and 500 ng of plasmid DNA were used per well. Plasmid DNA here comprised 450ng Luc*ATP8B1* promoter construct and 50ng Renilla pRL-TK internal control plasmid (Promega). For some stimulation experiments, 50ng pCI_hFXR and 50ng pCI_hRXR contructs were co-transfected. Twenty-four hours after transfection, cells were treated with DMSO, chenodeoxycholate, CDCA or taurocholate, TCA (Sigma Aldrich), respectively.

Reporter gene assay. Firefly and Renilla luciferase activities in cell lysates were determined 48 hours after transfection or 24 hours after stimulation with bile acids using dual luciferase system (Promega) and plate luminometer Glomax (Promega). All reporter gene assays were performed in triplicates and results are presented as the average value from at least three independent experiments normalized to the Renilla luciferase and pGL3-Basic activities.

Results

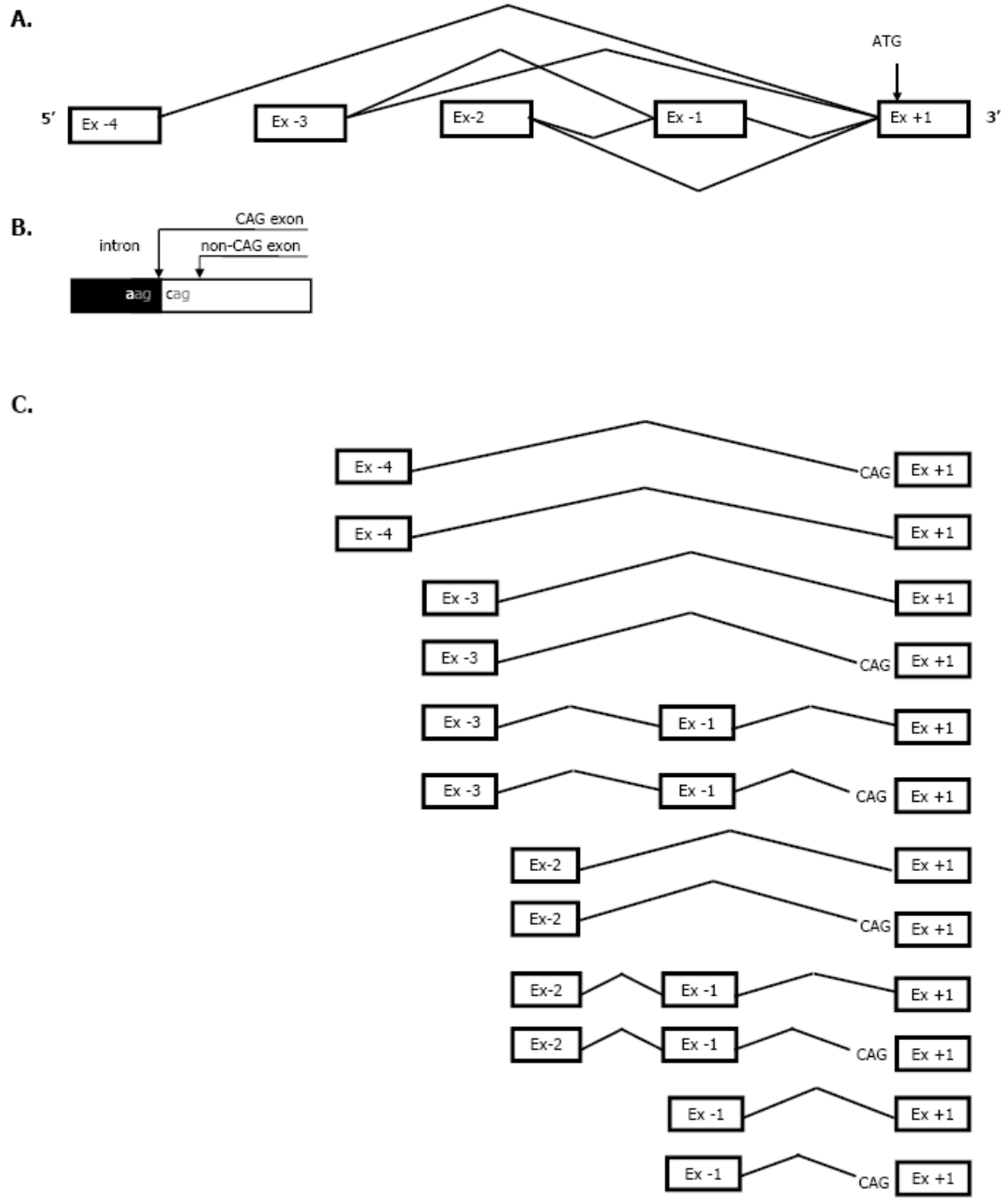
The 5´UTR of *ATP8B1* comprises four exons and spans a 71kb genomic region

ATP8B1 was previously shown to have 27 coding exons (13). Despite a previous attempt to attribute the region immediately upstream of the gene's first protein-coding exon as a promoter, it was not known whether the first exon (Ex +1) actually comprises the *ATP8B1* transcription start site. Therefore, we performed 5'RACE using primers in the coding exon +1 to search for the presence of additional 5' exon(s).

5'RACE using RNA extracted from normal human liver (n=6 adult, 1 infant) and intestinal (n=3) tissues revealed four novel untranslated exons located 29.952, 70.059, 71.125, and 71.748 kb upstream of exon +1. These new exons are 115, 93, 509, and 191 bp in size, respectively, and have been designated exons -1 to -4 (Ex -1 to Ex -4) (Figure1A). Six different splice variants were detected, comprising different combinations of novel untranslated exons, (Fig.1A). Due to the existence of two donor splice sites (tandem acceptors) at the 5'end of exon +1 (Ex +1), two different ways of splicing the untranslated exons to exon +1 are possible, as indicated in Fig. 1B. Indeed, two different variants for each splicing event including exon +1, differing from each other by only 3 bases (CAG), were observed. This subtle change represents a further source of 5'UTR variability generating in total, 12 mRNA isoforms. Fig. 1C

Fig.1 The heterogeneity of 5'UTR of *ATP8B1* gene. A. Four novel untranslaned exons of the *ATP8B1* gene were found and designated Ex -1, -2, -3 and -4. Six identified alternatively spliced variants are indicated by diagonal lines. **B.** The existence of two acceptor splice sites CAGCAG (tandem acceptors) at the 5' boundary of the first translated exon (Ex +1) of *ATP8B1* allows the generation of two different splice forms for each combination of upstream exons with Ex +1. **C.** Thus generated splice forms differ from each other by only three nucleotides CAG and C. give rise to 12 *ATP8B1* isoforms in total.

Figure 1



Six different splice variants were detected, comprising different combinations of novel untranslated exons, (Fig.1A). Due to the existence of two donor splice sites (tandem acceptors) at the 5'end of exon +1 (Ex +1), two different ways of splicing the untranslated exons to exon +1 are possible, as indicated in Fig. 1B. Indeed, two different variants for each splicing event

including exon +1, differing from each other by only 3 bases (CAG), were observed. This subtle change represents a further source of 5'UTR variability generating in total, 12 mRNA isoforms. Fig. 1C

Using commercially available RACE-Ready cDNA (Ambion) prepared from full-length capped mRNA from liver and intestinal tissue, several putative transcription start sites were identified: one each at the beginning of herein identified UTR exons except for exon -1 and several alternative transcription start sites within exon -3 (Figure 2). Transcription start site hot spot was located in the region between nucleotides -135 and -115 from the 3'end of exon -3. Fig.2B This region contains putative initiator sequence (*Inr*). RACE-Ready experiments also identified two other novel exons in the vicinity of exon -3 and exon -2. Exon -4 identified by classical RACE has not been detected using RACE-Ready cDNA. On the contrary, TSS at the beginning of exon -1 was only found by RACE, while using RACE-Ready cDNA exon -1 was a part of transcripts initiating further upstream of exon -1.

Figure 2

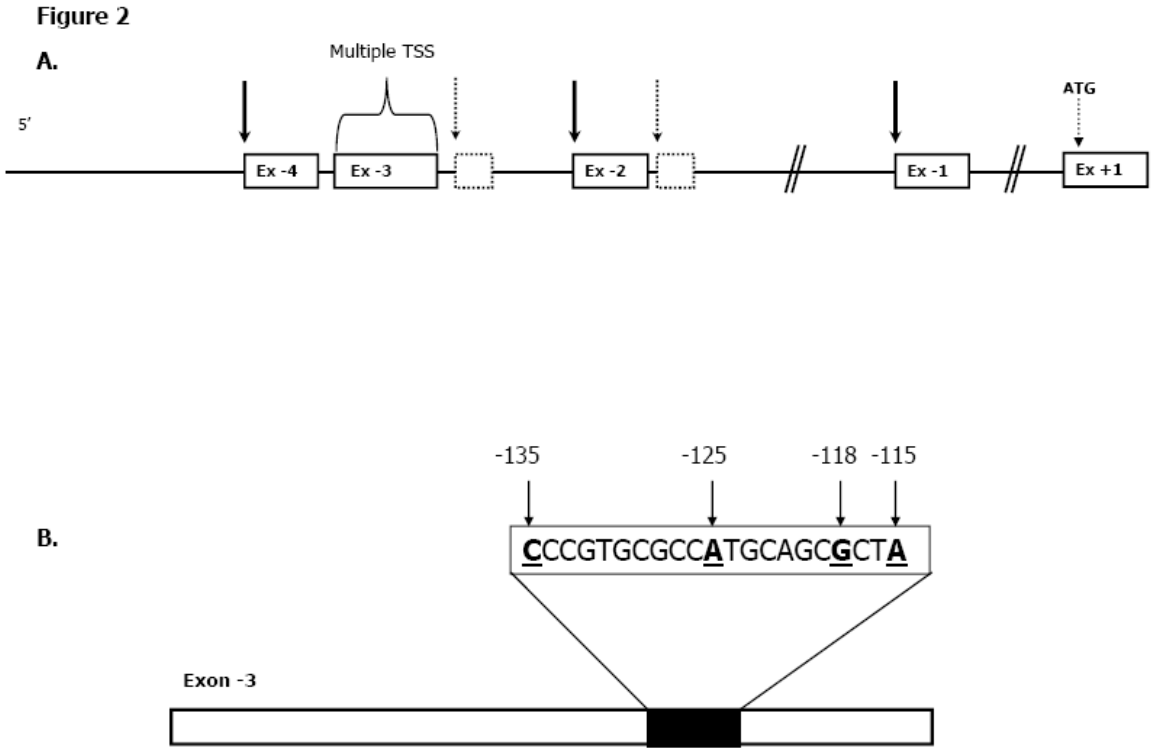


Fig. 2 Transcription start sites (TSSs) identified within 5'UTR of the *ATP8B1* gene.
A. Transcription of the *ATP8B1* gene originates from multiple TSSs (indicated by arrows); two additional TSS were identified in the beginning of rarely used exons, adjacent to exons -3 and -2 respectively (dashed boxes). Sixteen different TSS were found within the 509bp-long Ex -3 with the main cluster located between nucleotides -115 and -135 (B).

Consistent expression pattern of the novel ATP8B1 mRNA isoforms in the liver and intestine

To define the biological relevance of different *ATP8B1* transcripts, qRT-PCR was performed using RNA from normal human adult and one children's liver. Ten sets of specifically-designed probes covering all identified alternative exon-exon boundaries found by 5'RACE were used (Supplement. Tab.1). The results presented in Fig.3 demonstrate the abundance of 5'UTR splicing events relative to the protein coding region, represented by the exons +1/+2 boundary. qRT-PCR demonstrated the prevalent expression of transcripts containing exon -3 (Ex -3) directly spliced to exon +1 (Ex +1). These splicing variants, Ex -3/Ex +1 and Ex -3/CAG/Ex +1 (Fig. 3, variants III and IV), comprise almost 70% of total *ATP8B1* expression, whilst alternative form, Ex -3/Ex -1 (Fig. 3, variant V) comprises less than 10% of the total transcripts. Splicing variants Ex -1/Ex +1 and Ex -1/CAG/Ex +1 (Fig.3, variants IX and X) account for almost 30% and splicing events including exon -2 less than 4% of the total transcripts (Fig.3, variants VI, VII, VIII). The expression level of Ex -4/Ex +1 (Fig.3, variants I and II) , found by classical 5'RACE but not RACE-Ready cDNA varied among the samples, ranging from its complete absence to 3.4% of total gene expression.

The expression levels of two small rarely detected exons, located 91bp and 215bp 3' from exon -3 and exon -2 respectively, and identified only using RACE-ready cDNA, were in all experiments below 1% (data not shown).

Additional qRT-PCR experiments on a limited (n=3) number of intestinal samples did not exhibit any significant difference compared to the expression pattern detected in liver, with exon -3 proving to be the most prevalent 5'UTR exon expressed in both, liver and intestinal tissues.

Search in EST database (National Centre for Biotechnology and Information, http://www.ncbi.nlm.nih.gov/) revealed one *ATP8B1* transcript including exon -3 spliced to exon +1 missing the CAG triplet (GenBank accession: DR005588.1). This transcript does not include the protein coding exon +2, thus exon +1 is here spliced directly to exon +3. The predicted protein sequence demonstrated premature stop codon (TAA) at position 62.

We confirmed the existence of Ex +1/Ex +3 splicing event by PCR using cDNA templates isolated from liver and intestine. qRT-PCR using a specifically-designed probe for the Ex +1/Ex +3 boundary demonstrated minimal expression of this transcript in normal human liver and intestinal tissue, i.e. on average there was 20-fold lower expression of Ex +1/Ex +3 boundary containing transcript compared to the Ex +1/Ex +2 splicing variant (data not shown). The biological significance of the low abundance transcript remains unclear.

Also, our results did not confirm the existence of previously identified 909bp-long *ATP8B1* 5'exon (14).

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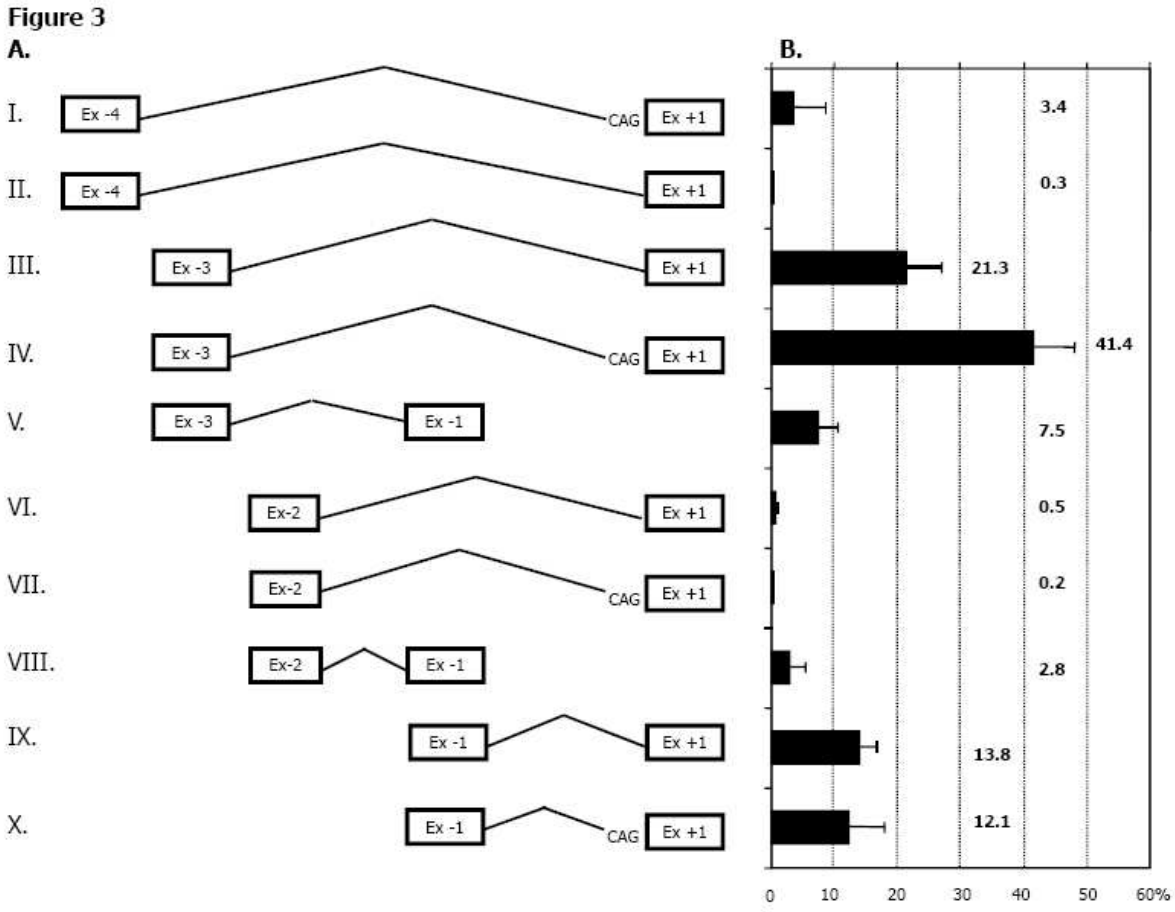

Fig. 3 Relative expression levels of different splicing forms assessed by qRT-PCR **A**. Diagram of 10 splicing events covered by individually designed probes (probe and primer complete sequence is in Supplement Table 1). Expression level was tested on normal liver tissues (n=7, **B**). The levels of transcripts containing a particular splicing event are presented as a relative value normalised for the expression of the protein coding region represented by Ex +1/Ex +2 boundary.

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Also, our results did not confirm the existence of previously identified 909bp-long *ATP8B1* 5'exon (14).

In silico identification of the putative promoters in the ATP8B1 gene
On the basis of the 5'RACE and qRT-PCR results, we predicted the major promoter region of *ATP8B1* to be located upstream of the cluster of transcription start sites within exon -3 (Prom 3,

Fig. 5) and some weaker promoters upstream of exons -2 and -1 (Prom 2 and Prom 1, Fig. 5). In agreement with our hypothesis, *in silico* analysis which employed three independent promoter prediction programs located putative promoter regions within a CpG island 70-72kb upstream of the protein coding exon +1 corresponding to the chromosomal location of the novel exon -3 identified in our 5'RACE experiments (Figure 5). Computer analysis did not predict the presence of promoter upstream of exon -1.

Figure 4

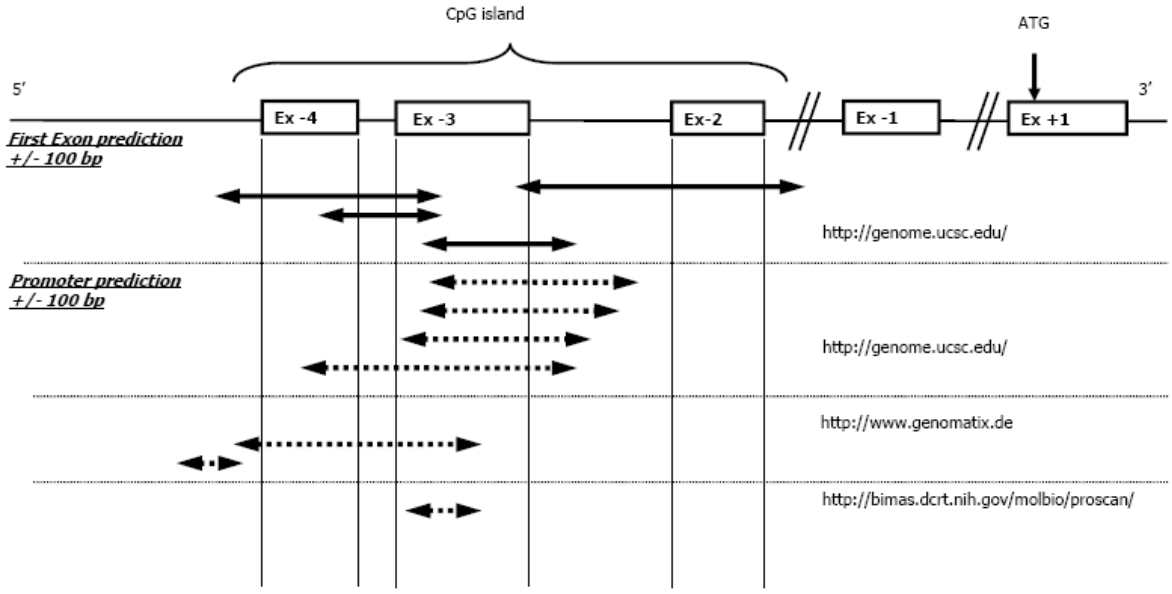


Fig 4. *In silico* analysis of first exon and promoter region for *ATB8B1* gene compared to the 5'RACE results. Four putative first exons were predicted by "First EF" computer prediction software (horizontal double-arrow lines) (107). Predicted exons correspond to the chromosomal location of the novel exons -2, -3 and -4 identified in the 5'RACE experiments. Three independent computer algorithms localised putative promoters (dashed horizontal double-arrow lines) in a CpG island 70-72 kb upstream of exon +1.

Experimental identification of the major promoter in the ATP8B1 gene

In order to identify whether the major promoter of *ATP8B1* resides upstream of exon -3, twelve constructs containing luciferase reporter (434bp to 3379bp in length) were prepared: 9 comprised the sequences upstream of the transcription start site (TSS) hot spot in exon -3 whilst the remaining three focus on regions upstream of exon -2 and exon -1. (Figure 5)

The luciferase assay results, summarised in Fig. 6, demonstrate the highest relative activity for the short 434 bp construct (Prom 1) situated immediately upstream of the major TSS cluster in exon -3. Longer constructs ranging from 747nt up to 3379nt (Proms 2-6) exhibited dramatically reduced promoter activity, approximately 25-30%, compared to the Prom 1 construct. Only minimal differences in the reporter assay were observed among Proms 2-6 constructs each other.

Removal of 434bp region resulted in complete lack of luciferase activity in all created constructs (Prom 7-9; Fig.6) thus emphasizing the importance of proximal P3 promoter in driving luciferase activity.

Figure 5

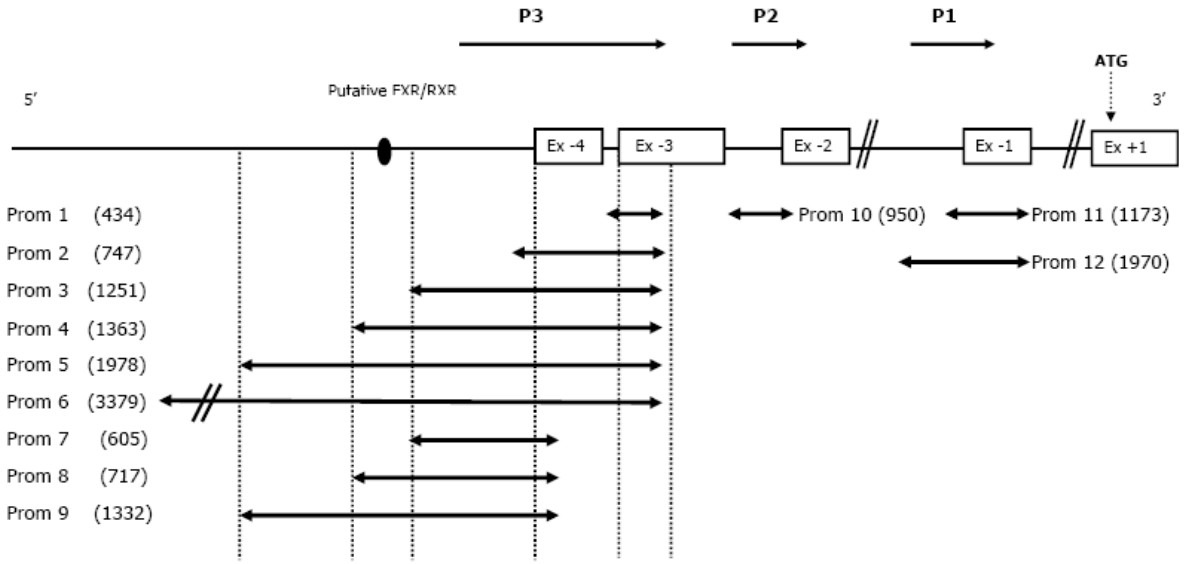


Fig 5. Schematic representation of the position and size of luciferase constructs used in promoter reporter assays. Nine luciferase constructs (Prom 1 to Prom 9) were designed to comprise the putative dominant promoter P3 , two constructs covered promoter P1 (Proms 11 and 12) and one promoter P2 (Prom 10). The position of the reporter gene constructs are indicated by horizontal double arrow lines. The number in brackets next to the construct name represents its size (nt). Prom 3 and Prom 4 were designed to include/exclude a putative FXR/RXR binding site indicated by black oval. Figure is not drawn to scale.

Promoter region upstream of exon -1 was not predicted *in silico* and putative TSS was detected only by classical 5'RACE, but not using RACE-Ready cDNA. Nevertheless, the 40kb distance between main promoter and exon -1 as well as the qRT-PCR data suggested the presence of alternative regulatory region in the vicinity of exon -1. Constructs which included the putative promoters upstream of exon -1 and exon -2 (Proms 10-12) showed basal luciferase activity which was <10% that of the Prom 1 construct upstream of exon -3 (Fig. 6).

The 5´UTR of ATP8B1 is highly conserved between human, mouse and rat

The multiple sequence alignment program ClustalW (http://www.ebi.ac.uk/clustalw) was used to align the genomic sequences of the 5' UTR of *ATP8B1/Atp8b1* from human, mouse and rat. A high degree of conservation was found in the region corresponding to exons -3 and -4: 83% and

82%, respectively, for a human-mouse and 66% and 69%, respectively, for a human-rat alignment (Fig. 7).

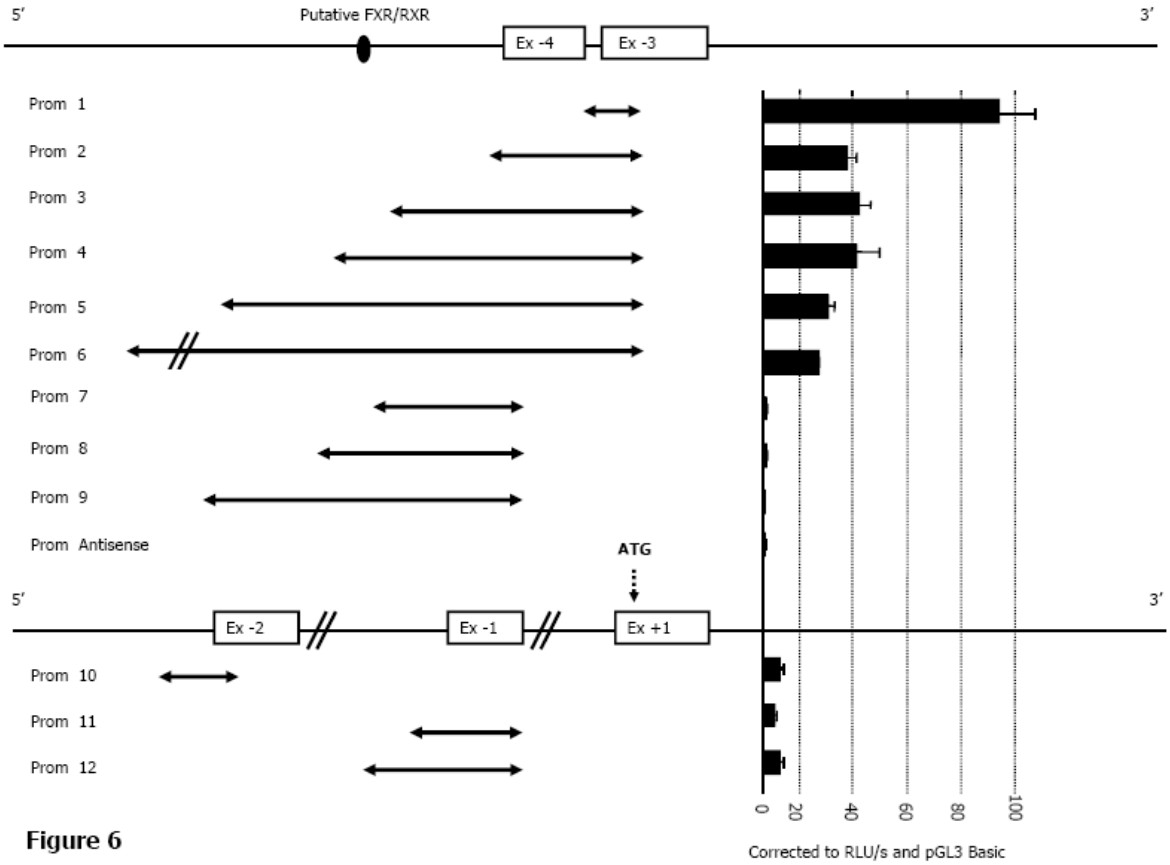


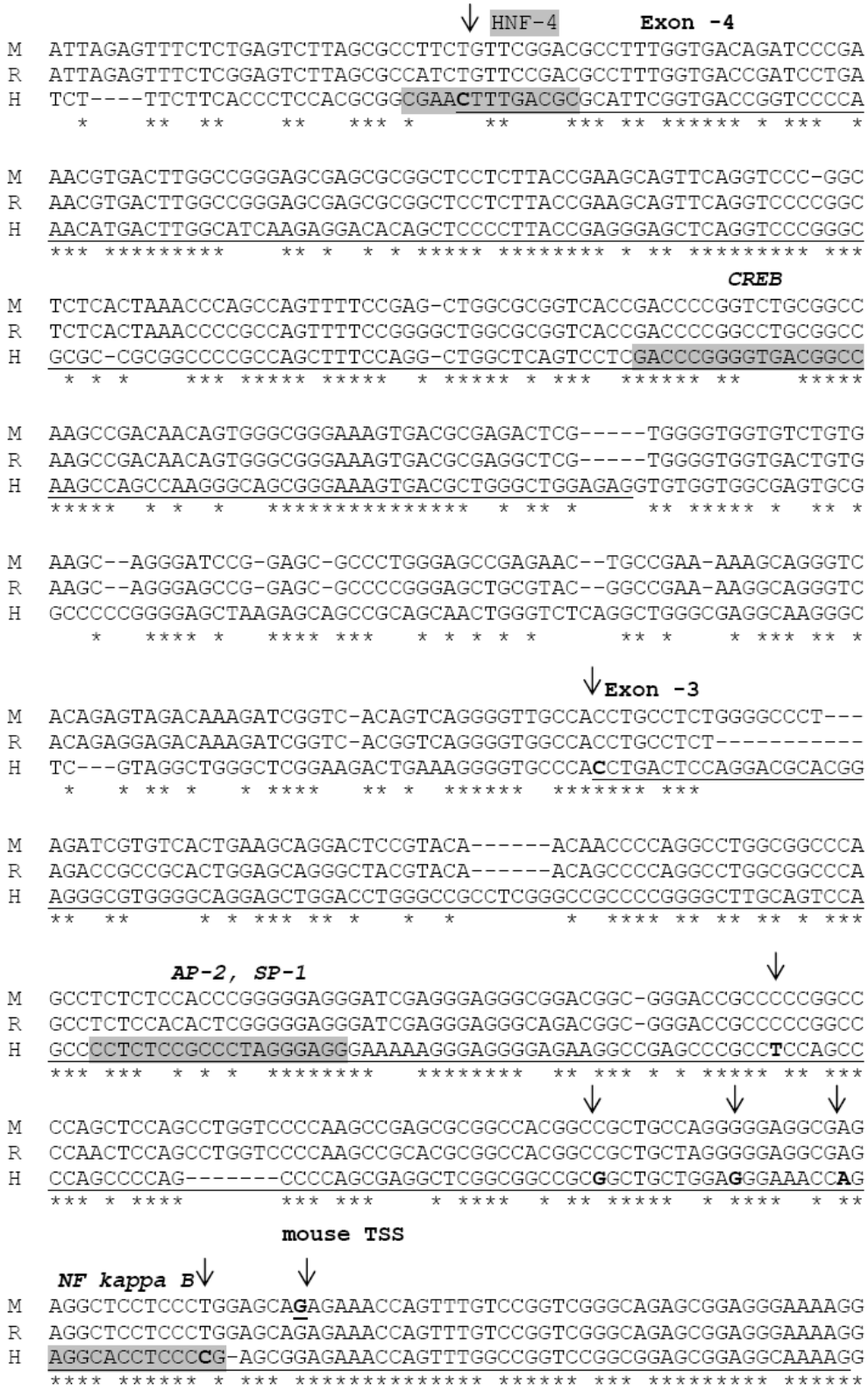
Fig 6. Functional analysis of *ATP8B1* promoter regions.
HepG-2 cells were transiently transfected with luciferase reporter gene constructs containing 12 different parts of putative promoter regions of *ATP8B1* depicted on the left. Antisense construct encodes the same region as Prom 5, but in antisense orientation. Luciferase activity for each construct is shown as relative light units per second (RLU/s) and represents the mean value calculated from 3–5 experiments. The data were corrected for the transfection efficiency using both internal control Renilla pRL-TK or pRL-null and pGL3 Basic activity.

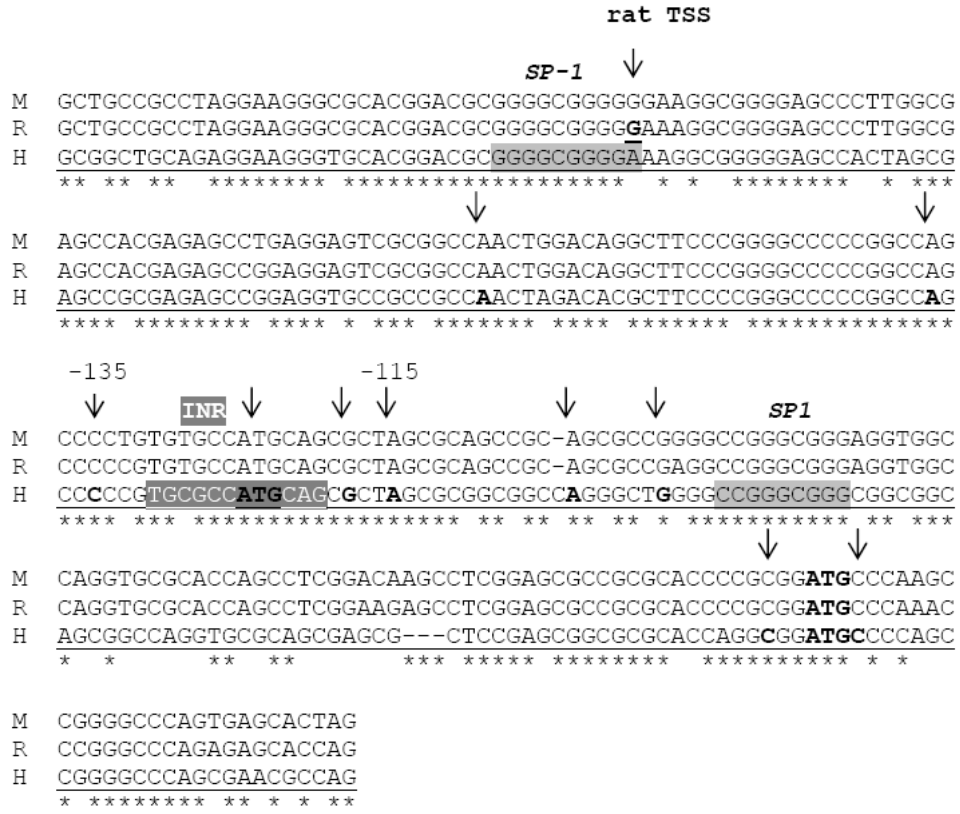
Transcription factor binding sites present in the ATP8B1 promoter region
Promoter region sequences of *ATP8B1* were further analysed using web-based versions of MatInspector (www.genomatix.de), AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and TFSearch 1.3 (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) to check for the presence of putative transcription factor (TF) binding sites.
No consensus TATA or CAAT boxes were found in the proximal P3 promoter of the *ATP8B1* gene. On the other hand, several putative binding sites for non-specific, general TFs (Sp1, AP-2,

NFκB) were identified here. No liver or intestine specific TF binding sites were found in the vicinity of the TSSs cluster of exon -3 (Fig. 7).
A putative farnesoid X receptor (FXR/RXR) binding site was located only by Genomatix software 807bp upstream of the exon -4 (Fig. 5). However, this site, **GAGT**G**A**c**TGACC**A, does not correspond to any known consensus FXR binding sequence and is not conserved between human, mouse and rat.

Figure 7

A. Exons -3 and -4, proximal promoter P3





B. Exon -2

aagttctgaagtgtctgcccaacttttccgacggaggcgtgtgcctgtggtccctgccgggggttggtggc
tggttgcgggggggggcggtgctcggagcgcagatccgtgacggtt**GTACGGGGGAGACTGGATTGCAGTA**
AGGATGCAAATTGGGGAAGGGGGATTGACACGCAGAAGTAAGGAGGACTTGGGCCGCGCTTCTGCCCGgtg
agtacccgctcaaccctacccgacccccttggcctgcacgcgctgcccttgggggctgcaagctcttcctg
ggtagctccagacgcc

C. Exon -1 and part of promoter P1 containing Alu sequence

Tgtcactatacatttgccaaaccccacacaacaaagagtgaaccctagtgaactgtggacttcatgtaaac
tagggactttagttacaatatgtcattactggggccgggcacattggctcacacctgtaatctcagcactt
cgggggggctgacgcgggcagatcacttgaggtcaggagttcaagaccagcctggccaacatggtgaaaccc
tgtctctactgaaaatacaaaaattagccaggcgtgttggcgggcacctgtagtcccagttactcaggagg
ctgaggcaggagaatcgcttgaacctgggaaatgggggttgcagtgagccgagatcacattactgcactac
agcctgggtaacagagtgagaccctgtctcaaaagaaaaaaaaaatatatatatatatatgtgtgtgtgtg
tgtatctcattattggctcatcagttgtaacaaatgtgccactctaatgcaagatgtgaacagcgtacact
aaggcacggggctgaaaggatatgtggggattgccactccatttttccgtaaacctaaaactattcagtaa
taatcctttttttttttttttttttttttaacag**TCTTGCTCTGTCACCAGGCTGGAGTGCAGTGGCGCAA**

Fig 7. Putative transcription factor binding sites and conservation of *ATP8B1* 5'UTR. A. Sequence alignment using ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw/). High level of conservation among mouse (M), rat (R) and human (H) genome was detected for exon -3 and exon -4 (conserved nucleotides indicated by stars). Putative Sp-1, Ap-2, NFκB transcription factor binding sites predicted in exonic/promoter region P3 (exon -3 and proximal upstream sequence). Putative CREB and HNF-4 binding sites were identified within a distal part of promoter P3 corresponding to exon -4 sequence. Exonic regions are underlined, transcription start sites are indicated by arrows and bold letters and putative transcription factor binding sites by grey boxes. Initiator element sequence encompasses the main TSS cluster (nt-115 and -135 of exon -3). Two upstream ATGs are in bold. Sequences of Exon -2 **(B.)** and Exon -1 **(C.)** containing AluS consensus sequence. Exonic region is highlighted in bold. Alu sequences are underlined.

Stimulation with bile acids

To investigate the effect of bile acids on the regulation of *ATP8B1* expression, HepG2 cells were transfected with the Prom 3, Prom 4, and Prom 6 *ATP8B1* luciferase constructs comprising main promoter P3 and incubated in the presence or absence of sodium chenodeoxycholate (CDCA) and taurolocholate (TC) for 24 hours. Prior to the luciferase experiments, the expression levels of *ATP8B1, ABCB11, SHP* and *CYP7A1* were evaluated by qRT-PCR before and after CDCA treatment to assess the normal response of the cells to the bile acid stimulation. While *ATP8B1* levels remained constant, *ABCB11* and *SHP* mRNAs were up-regulated and *CYP7A1* mRNA down-regulated in the presence of CDCA, demonstrating a functional FXR→SHP→*ABCB11* regulatory pathway in our HepG2 cell line.

In agreement with the above demonstrated unchanged mRNA expression of *ATP8B1* under CDCA stimulation, none of the luciferase constructs demonstrated a significant change of promoter activity in HepG2 cells after CDCA (Fig.8A) or TCA (data not shown) treatment.

Since HepG2 cells do not express the sodium-taurocholate co-transporting polypeptide NTCP, the main uptake bile acid transporter in the basolateral membrane of hepatocyes, all experiments were repeated with rNtcp-HepG2 cells, a HepG2 cell line stably expressing rat Ntcp. To minimise the effect of bile acids present in FBS, the cells were cultured in charcoal-stripped FBS which removes >90% of bile acids present in the serum (11). Stimulation of rNtcp-HepG2 cells with CDCA resulted in no significant change in promoter activity of all tested luciferase constructs (data not shown).

We finally assessed the effect of bile acids on the *ATP8B1* expression in the presence of FXR/RXR by co-transfecting 250ng of the *ATP8B1* luciferase constructs Prom 3, Prom 4 or Prom 6 into rNtcp-HepG2 cells along with 50ng of pCI plasmid containing human retionid X receptor α (pCI_hRXR α) and 50ng of pCI plasmid containing human farnesoid X receptor (pCI_hFXR). The cells were stimulated with 0, 10 and 25 μmol of CDCA. In agreement with previous experiments, no significant change in *ATP8B1* promoter activity was observed (Fig

8B). These results indicate no direct link between FXR, bile acids and the *ATP8B1* major promoter.

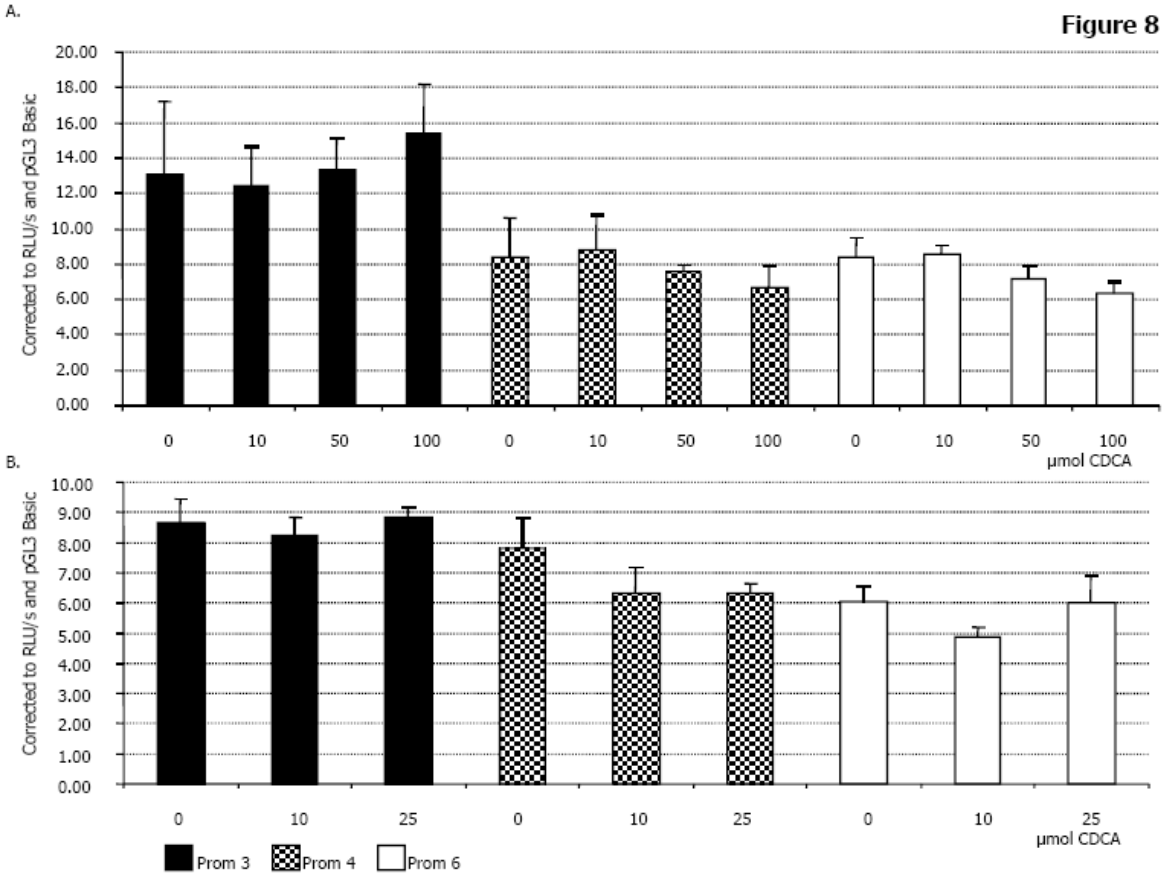


Fig. 8 The *ATP8B1* promoter activity in cells stimulated with bile acids. A. HepG-2 cells were transiently transfected with three representative *ATP8B1* promoter/reporter gene constructs (Prom 3, 4 and 6) previously characterised (Fig.7) and stimulated with 0, 10, 50 and 100 µmol CDCA for 24 hours. All constructs comprise proximal, 434nt-promoter P3, Prom 4 includes putative FXR binding site identified by MatInspector computer analysis software, and Prom 6 represents the largest construct containing 3379nt of 5'flanking region. **B.** HepG-2 cells stably expressing rat sodium-taurocholate co-transporting polypeptide (rNtcp) were transiently transfected with constructs Prom 3, 4 and 6 together with 50ng of pCI_hRXRα and 50ng pCI_hFXR plasmids and treated with 0, 10 and 25 µmol CDCA for 24 hours. No significant change in activity was detected after stimulation.

Discussion

The *ATP8B1* gene was previously known to be composed of 27 protein-coding exons. Mutations in *ATP8B1* were shown to underlie a spectrum of low γGT cholestases (PFIC1, BRIC1, ICP), now called ATP8B1 (FIC1) deficiency (13, 15-17).

Even though the gene is important biologically and clinically, our knowledge of its regulation remains limited.

This study shows that previously published transcription start site (TSS) of *ATP8B1* is not significant and that its expression is driven by a highly structured 5'UTR. Four alternatively spliced untranslated exons located in a considerable distance upstream of the first protein coding exon generate 10 different transcripts. The existence of a previously published untranslated exon (14) was not confirmed. The transcription of the gene is driven by three newly identified promoters (P1, P2 and P3; Fig.5) located upstream of exons -1, -2 and -3. In liver, the promoters P1 and P2 play only minor role under the physiological conditions (Fig.6). Promoter P3 is located within CpG island and was proved to be the essential regulatory element responsible for 70% of total gene expression. The multiple transcription start sites hot-spot was identified within nucleotides -115 to -135 from the 3'end of exon -3. This region contains putative initiator element (*Inr*) (18, 19) and is encompassed by Sp1 binding sites. (Fig.7).

Proximal sequence of promoter P3 is promiscuously serving both as exonic and promoter region. Interestingly, this region was proved to be a driving force of promoter P3 and longer constructs containing the upstream sequence all displayed approximately 3-fold less activity (Fig.5). Constructs missing the proximal 434nt P3 promoter demonstrated complete lack of promoter activity comparable to the construct in antisense orientation. This observation thus emphasizes the principal role of this 434nt region in driving the transcription of the *ATP8B1* gene and confirms our qRT-PCR data (Fig. 3) demonstrating only minimal expression for splicing variant Ex -4/Ex +1, which cannot use 434bp proximal P3 promoter region.

The importance of proximal P3 promoter was further confirmed by sporadic use of transcription start sites located further upstream of the main TSS cluster between
nucleotides -115 and -135 (Fig.7). The dominant *ATP8B1* promoter thus displays the typical features for promoters of housekeeping genes: TATA-less, GC-rich sequence with multiple transcription start sites. In addition, only non-specific putative transcription factor binding sites, Sp1, AP-2 and NFκB, were identified within the proximal promoter P3. (Fig. 7)

These data are in agreement with the ubiquitous expression of ATP8B1 (FIC1) and its putative complex role in maintenance of apical membrane structure (7).

Since *ATP8B1* deficiency is primarily characterised by cholestatic phenotype, most studies focused on its role in cholestasis. Some of them attempt to attribute the cholestatic phenotype to a defective FXR signalling (8, 10, 11), but other studies suggest that impaired FXR activity is secondary due to cholestasis and, as such, is not responsible for PFIC1 phenotype (4, 9, 12).

It has been previously shown that there is no change in *ATP8B1* mRNA levels in cholestatic patients (20). In our experiments, stimulation of HepG2 cells with CDCA indicated no change in *ATP8B1* mRNA levels, despite the changes in the expressions of *Cyp7A1* and *ABCB11*. More, the CDCA treatment of HepG2 or rNTCP-HepG2 cells expressing various *ATP8B1* promoter constructs (the largest one 3379nt long) has not demonstrated any significant changes in luciferase activity.

No change of the *ATP8B1* gene expression in response to CDCA treatment is in agreement with its ubiquitous expression, including both organs involved in bile acid circulation and those apparently unrelated to bile acid homeostasis such as lung, heart, placenta and stereocilia of inner ear (13, 21, 22). Consistent with a widespread expression of ATP8B1 (FIC1), PFIC1 phenotype includes hepatic and extrahepatic features. This suggests uniform, bile acid-independent mechanisms regulating ATP8B1 expression and function across different tissues.

The function of ATP8B1 (FIC1) protein in cell physiology and its role in cholestasis still remains to be elucidated. The protein apparently plays a critical role in structure and/or organisation of apical plasma membrane (Paulusma 2006, Verhulst 2010), a familiar factor affecting critical cellular processes including transport and signalling. Thus the loss of canalicular membrane asymmetry may help to explain symptoms associated with ATP8B1 deficiency across organs.

On the contrary, some organs expressing FIC1 (e.g. kidney, lungs) are apparently unaffected in PFIC 1 patients. Absence of clinical symptoms on one hand, and no correlation between severity of phenotype and *ATP8B1* mRNA levels in various affected organs on the other hand, supports the hypothesis of involvement of other P4 ATPases that are ubiquitously, though at variable levels, expressed across organs. (23). Hence, the essential prerequisite for better understanding the relation between ATP8B1 and other P4 ATPases is the detailed characterisation of their tissue-specific expression pattern and role in cell physiology.

Genes regulated by alternative promoters are common in humans. Multiple promoters can be used according to environmental conditions or developmental stage to ensure the tissues-specific or spatio-temporal expression of corresponding isoform. Various mRNA isoforms may also interact to achieve a transcriptional repression of an alternative transcript (24, 25).

It was shown, that from several alternative mRNA isoforms, one can be ubiquitously used among the cell types, whereas the others may be limited to a small number of tissues (26-29). This might be the case of *ATP8B1* alternative transcripts. Even though our pilot study does not support such tissue specific regulation of *ATP8B1* at transcriptional level in tested samples, further research is needed to characterise the role of all three promoters in different organs and under varying conditions and to address the involvement of post-transcriptional control mechanisms..

In a view of a complex structure of the *ATP8B1* gene and highly variable mRNA levels across the cell types, RNA stability and post-transcriptional control appears to be more important in *ATP8B1* regulation than previously expected.

Our data demonstrate significant difference between the activity observed for promoter upstream of exon -1 (P1) versus promoter upstream of exon -3 (P3). However, this difference was not followed by the similar ratio in mRNAs levels of the transcripts suggesting the different efficiency of post-transcriptional processing. Whereas the reporter gene activity mediated by promoter P1 was 15-fold lower than that of the principle promoter P3 (Fig. 6), the mRNA levels of corresponding transcripts displayed only 3-fold difference (Fig. 3). The discrepancy observed indicates that *ATP8B1* transcripts are apparently subjects of extensive post-transcriptional regulation.

5’UTRs are known as key mediators of post-transcriptional control. The mechanisms of UTR-mediated regulation comprise, among others, stable secondary structures including those formed by repetitive sequences such as Alu and upstream open reading frames (30).

Alternatively spliced exon -1 is an exonized truncated AluS element with the promoter P1 containing complementary Alu sequence. Alu sequence embedded in 5’UTR was demonstrated to modulate both transcription and translation (31, 32). Besides formation of stable secondary structures, Alu sequence is a source of hormone response elements, which involvement in gene regulation *in vivo* remains to be elucidated (33, 34).

Upstream Open Reading Frames (uORF) were recently recognized as potent, yet not fully understood, modulators of transcriptional and translational efficiency. Whereas some uORFs appeared not to affect gene expression (35, 36), others can do so by several distinct mechanisms.

The uORF-mediated inhibition of mRNA stability and uORF-mediated translational repression are principal mechanisms of such control.

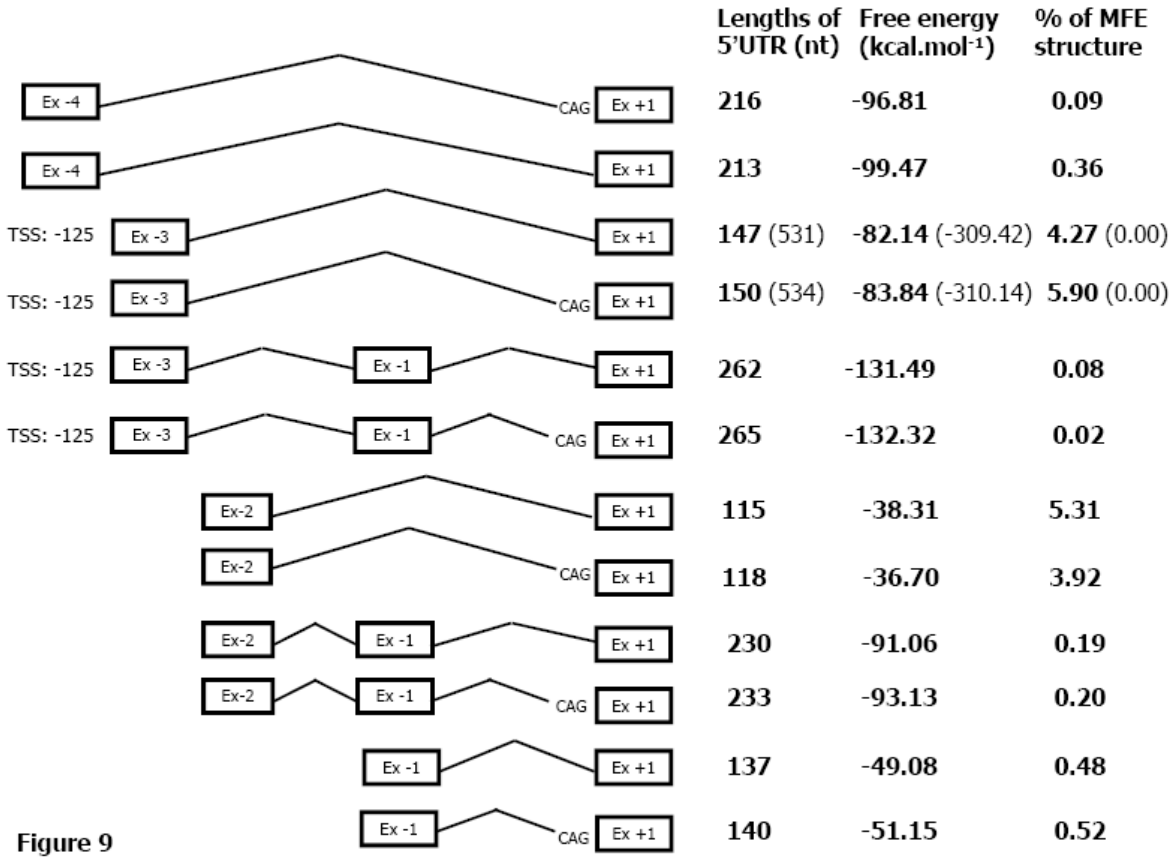


Fig. 9 Thermodynamic properties of identified 5'UTR isoforms. The comparison of the 5'UTR length and RNA secondary structure free energy and percentage of MFE for all identified *ATP8B1* 5'UTR isoforms schematically depicted on the left. Putative secondary RNA structures predicted using RNAfold web tool (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) are summarised in Supplementary Fig. 1. Prediction for the most frequent isoforms initiating at exon -3 was calculated using TSS at position -125. Data in brackets (row 3 and 4) represent data for TSS at position -509.

Altering the stability of mRNA by its ability to trigger the nonsense-mediated mRNA decay (NMD) (37) represents a process that could be potentially involved in the regulation of *ATP8B1* mRNAs isoforms. In this study, we identified *ATP8B1* transcripts that differ in their leader sequences and in the presence of putative upstream start codons AUG (uAUG) (Fig 7). Whereas no uAUG was found within transcript containing exon -1, two uAUGs and two uORFs were identified within the prevalent transcript containing exon -3. Recently, the inhibition of downstream ORF by upstream located uAUG was demonstrated for ABCC2 (MRP2) gene (38). Similar mechanisms, a uORF-mediated repression of a major ORF and NMD-dependent mRNA

instability, were proved as a key regulators of *IFRD1* gene expression in resting human kidney epithelial cells cells. During stress response, both mRNA and protein levels were increased as a result of mRNA stabilisation due to reduced translation of uORF (39).

Heterogeneity, high GC content and unusual length of *ATP8B1* 5'UTR indicate its role in the regulation of the gene expression. In addition to abovementioned mechanisms, formation of stable secondary structures that may impede the progress of the scanning ribosome is suggested. It was demonstrated that the extent to which scanning is affected is influenced by the size and position of the secondary structure(s) towards the 5'cap of the mRNA species, i.e. an alternative transcript with a shorter version of the 5'UTR is frequently translated more efficiently than the one with a longer 5' region (40-42). Likewise, the stem-loop structure located in a considerable distance from 5'cap will require higher free energy in contrast to a closer situated formation to affect the access of a pre-initiation complex to the mRNA. (43, 44). Figure 9A summarizes all identified *ATP8B1* mRNA isoforms, the length of their 5'UTRs and calculated free energy and the frequency of the minimum free energy (MFE) structures (45). Drawings of the MFE structures are depicted in Supplement material, Fig.1.

Because of complexity of the post-transcriptional and translational machinery, both balance and interplay between many factors involved in these processes together with spatial arrangements of mRNAs are essential for the optimal regulation of gene expression. Thus the 5'UTR-mediated translational repression can be overcome by increased levels of initiation factors as demonstrated for example for eukaryotic cap-binding translation initiation factor 4E (eIF4E) in several tumour types (46-48). Despite the fact that most studies refer preferentially to pathological processes, it is highly likely that similar mechanisms are functional at physiological levels.

Present study provides a fundamental data about the complexity of *ATP8B1* gene regulation. Newly identified *ATP8B1* mRNA isoforms differ in 5'UTRs and both transcriptional and translational efficiency. The basal expression of the *ATP8B1* gene in liver and intestine is driven by promoter with house-keeping like properties. Bile acid stimulation does not influence its expression, which is in line with both hepatic and extra-hepatic features of FIC1 deficient patients and suggests the bile-independent mechanism of tissue damage.

In the future, we aim to address the role of post-transcriptional processing in the modulation of *ATP8B1* expression and detail analysis of the role of all three promoters in different tissues and under various conditions.

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3.2. Hereditary jaundice

3.2.1 Dual hereditary jaundice

Mutations in *ABCC2* encoding MRP2 protein cause Dubin-Johnson syndrome (DJS), a disorder defined by autosomal recessive conjugated hyperbilirubinemia with a mild, benign course. Most underlying mutations result in complete absence of MRP2 protein in the canalicular membrane of hepatocytes (84). Rarely the defective protein is expressed and available for immunohistological detection (85).

Our aim was to establish the molecular diagnosis in a 3-year-old male with atypical, intermittent, predominantly unconjugated hereditary hyperbilirubinemia.

Though the conventional immunohistology and confocal-laser scanning microscopy showed complete absence of MRP2 in the liver, the inconclusive clinical presentation (mixed predominantly unconjugated jaundice and the lack of melanin-like liver pigment typical for DJS) indicated a multiple genetic defects.

Mutational analysis revealed two novel heterozygous mutations in the *ABCC2* gene.

Intermittent unconjugated hyperbilirubinemia reported in the patient's mother suggested an additional defect in a bilirubin excretory pathway. Analysis of a gene encoding the "bilirubin-conjugating" enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1), mutated in Gilbert syndrome (GS), demonstrated homozygosity for the A(TA)7TAA allele of the TATAA box of the *UGT1A1* promoter and for a -3279T>G mutation in the phenobarbital-responsive enhancer module.

Gilbert syndrome has recently come to be considered a metabolic variation. It manifests clinically in approximately two-thirds of the homozygotes for the *UGT1A1* A(TA)7TAA allele. This indicates that additional factors are required for full penetrance. We proposed that DJS was a promoting factor for full expression of the GS phenotype and supported our hypothesis by a retrospective analysis of a large Israeli cohort of DJS patients (108).

Dual hereditary jaundice: simultaneous occurrence of mutations causing Gilbert's and Dubin-Johnson syndromes

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Gastroenterology. 2005 Jul;129(1):315-20.

Abstract

Background & Aims: Dubin–Johnson syndrome is recessively inherited, conjugated hyperbilirubinemia induced by mutations in the *ABCC2/MRP2* gene encoding the canalicular transporter for conjugated bilirubin. Gilbert's syndrome is recessively inherited, unconjugated hyperbilirubinemia caused by decreased conjugation rate of bilirubin associated mostly with homozygous A(TA)7TAA variant of the TATAA-box in the *UGT1A1* gene promoter.

Our aim was to establish the molecular diagnosis in a 3-year-old male with atypical, intermittent, predominantly unconjugated, hyperbilirubinemia. *Methods:* 99mTc-HIDA cholescintigraphy was used for imaging the biliary tree. Expression of ABCC2/MRP2 protein in hepatocytes was investigated immunohistochemically. *UGT1A1* and *ABCC2/MRP2* genes were sequenced from genomic DNA, and the mutations were verified by fragment analysis, sequencing the cloned exons, and restriction fragment length polymorphism. *Results:* Cholescintigraphy revealed delayed visualisation of the gallbladder. A brown granular lipopigment differing from melanin-like pigment reported in Dubin–Johnson syndrome was present in hepatocytes, but, otherwise, liver histology was normal. ABCC2/MRP2 protein was not detected on the canalicular membrane of hepatocytes, and 2 novel mutations were found in the *ABCC2/MRP2* gene: a heterozygous in-frame insertiondeletion mutation 1256insCT/delAAACAGTGAACCTGATG in exon 10 inherited from the father and a heterozygous deletion 4292delCA in exon 30 inherited from the mother. In addition, the patient was homozygous for -3279T>G and A(TA)7TAA mutations in the *UGT1A1* gene promoter. *Conclusions:* Our patient represents a case of digenic mixed hyperbilirubinemia - a distinct type of constitutive jaundice resulting from coinherited defects in *ABCC2/MRP2* and *UGT1A1* genes.

The excretory pathway for bilirubin consists of 2 sequential steps: glucuronidation of unconjugated bilirubin catalyzed by UDP-glucuronosyltransferase 1A1 (UGT1A1) and biliary secretion of conjugated bilirubin via ABCC2/MRP2, the bilirubin export pump expressed on the canalicular membrane of hepatocytes. Impairment of bilirubin glucuronidation because of reduced activity of UGT1A1 causes Gilbert's syndrome (GS; On-line Mendelian Inheritance in Man database [OMIM] No. 143500), the most frequent hereditary hyperbilirubinemia affecting 5%–10% of the white population.[1,2] The disorder is characterised by mild, chronic, fluctuating, unconjugated hyperbilirubinemia with serum bilirubin levels up to 6.0 mg/dL. Mutations in the *UGT1A1* gene are responsible for the molecular basis of GS.3,4 An insertion of 1 TA repeat in the TATAA box of the *UGT1A1* gene promoter, named also *UGT1A1*28* allele, is the almost exclusive mutation present in the homozygous state in 11%–16% of European and North American populations.[4,5] Dubin–Johnson syndrome (DJS; OMIM No. 237500) is a rare, benign, predominantly conjugated hyperbilirubinemia with typical deposits of melanin-like pigment

within hepatocyte lysosomes. 99mTc-HIDA cholescintigraphy is characterized by prolonged homogenous visualisation of the liver with delayed filling of the gallbladder. [6,7] Total urinary porphyrin output is normal, but the ratio of urinary coproporphyrin isomers I and III is shifted from 1:3 to 4:1. DJS has an autosomal recessive mode of transmission, and mutations in the *ABCC2/MRP2* gene are responsible for the phenotype (Table 1).

Presumably because of the frequency of GS, a significant number of the individuals affected with DJS present with predominantly unconjugated hyperbilirubinemia. Combination of DJS with GS was suggested as an explanation for such atypical variant of DJS in the case reported by Tanikawa and Abe.[8] In our report, we present the first case of this digenic hyperbilirubinemia proven at the molecular level.

Table 1. Mutations in the *ABCC2/MRP2* Gene Associated With Dubin–Johnson Syndrome

Mutation	Exon	Consequence	Reported by
298C>T	3	R100Stop	Shoda J et al, Hepatol Res 2003;27:323–326.
1031 + 4A>G	8	Complex splicing	Mor-Cohen R et al, Hepatol Res 2005;31:104–111.
1815 + 2T>A	13	Skipped exon	Wada M et al, Hum Mol Genet 1998;7:203–207.
1967 + 2T>C	15	Skipped exon	Kajihara S et al, Biochem Biophys Res Commun 1998;253:454–457.
2026G>C	16	G676R	Wakusawa S et al, J Hum Genet 2003;48:425–429.
2125T>C	17	W709R	Machida I et al, Hepatol Res 2004;30:86–90.
2302C>T	18	R768W	Wada M et al, Hum Mol Genet 1998;7:203–207.
2439 + 2T>C	18	Skipped exon	Wada M et al, Hum Mol Genet 1998;7:203–207.
3196C>T	23	R1066Stop	Paulusma CC et al, Hepatology 1997;25:1539–1542.
3449G>A	25	R1150H	Mor-Cohen R et al, J Biol Chem 2001;276:36923–36930.
3517A>T	25	I1173F	Mor-Cohen R et al, J Biol Chem 2001;276:36923–36930.
3928C>T	28	R1310Stop	Tate G et al, Genes Genet Syst 2002;77:117–121.
4145A>G	29	Q1382R	Toh S et al, Am J Hum Genet 1999;64:739–746.
4175delGGATGA	30	Del RM	Tsujii H et al, Gastroenterology 1999;117:653–660.

Materials and Methods

Case Report

Our patient is a 3-year-old boy born after a full-term and uneventful pregnancy as the first child of unrelated parents. The family history was negative. Neonatal jaundice was managed by phototherapy for 5 days. The boy was breast-fed for 3 months, thrived well, and had no jaundice. At the age of 2 years, the patient was examined for allergic exanthema after administration of amoxicillin/clavulanate (potassium salt), and mild hyperbilirubinemia was detected (total bilirubin 2.1 mg/dL, direct bilirubin 0.5 mg/dL). Serologic screening tests for hepatotropic viruses (hepatitis A, B, and C; Epstein–Barr virus; and cytomegalovirus) were negative. Other laboratory findings were within the normal range. Bile duct obstruction was excluded by ultrasonography, and serum bilirubin level dropped spontaneously into the normal range. One year later, fever, vomiting, and scleral icterus appeared; increased serum levels of both total (5.6 mg/dL) and direct reacting (2.3 mg/dL) bilirubin were detected; and the boy was admitted to the hospital. Complete blood count was within the normal range, total serum bilirubin level decreased to 3.0 mg/dL and direct reacting fraction to 0.7 mg/dL. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase, α1-antitrypsin, copper, ceruloplasmin and thyroid hormones, sweat chloride concentration, and screening biochemistries for metabolic diseases were all within the normal range. Urinary coproporphyrin output was normal (40 μg/24 hours), but the level of isomer I was increased to 94%. There was no evidence of infection with hepatotropic viruses. No pathology was observed on ultrasonography of the liver and bile ducts. 99mTc-HIDA cholescintigraphy revealed prolonged

visualisation of the liver and delayed filling of gallbladder. Laboratory examination of the parents, based on their informed consent, revealed mild, unconjugated hyperbilirubinemia in the mother (total bilirubin, 1.7 mg/dL).

Histology and Immunohistochemistry

Histology. Liver biopsy specimens were stained with H&E, periodic acid Schiff (PAS) method, and silver ammonium complex (Masson's method). Antibodies. The anti-MRP2 mouse monoclonal antibody (clone M2III-6) was purchased from Kamiya (Seattle, WA). The rabbit polyclonal anti-human carcinoembryonic antigen (CEA) antibody, which recognizes CEA-related adhesion molecule 1 (CEACAM1) on biliary canaliculi of human liver, was purchased from DAKO (Glostrup, Denmark) together with the EnVision Peroxidase Kit and LSAB+ Kit. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody and Cy5-conjugated goat anti-mouse antibody were obtained from Jackson (West Grove, PA).

Immunohistochemistry.

For immunohistochemical procedures, 5-μm-thick sections cut from formalin-fixed, paraffin-embedded tissue samples were deparaffinized and pretreated with 2.7% hydrogen peroxide and 0.1% sodium azide. The slides were incubated with primary mouse anti-MRP2 monoclonal antibody diluted to 1:20 with Tris-buffered saline (TBS) containing 5% fetal calf serum and primary rabbit anti-CEA polyclonal antibody, diluted to 1:1000 in the same buffer. The EnVision Peroxidase Kit and the LSAB+ Kit were used to visualize sections incubated with primary mouse monoclonal antibody and primary rabbit antibody, respectively. The chromogen 3,3-diaminobenzidine from FLUKA (Buchs, Switzerland) was applied to all sections, and counterstaining was performed with Harris's hematoxylin. As a positive control, sections of an adult liver with minimal hepatopathy without cholestasis were stained. Liver sections incubated without primary antibodies were used as negative controls.

Confocal laser scanning microscopy. Microsections were deparaffinized and pretreated as described in the previous section. Double immunolabelling was performed as follows: Slides were simultaneously incubated with primary mouse anti-MRP2 monoclonal antibody (dilution 1:40) and primary rabbit anti-CEA polyclonal antibody (dilution 1:2000), both diluted with TBS containing 5% fetal calf serum at 4°C for 12 hours. Subsequently, simultaneous incubation of specimen with FITC/Cy5-conjugated secondary antibodies, both diluted to 1:100 with phosphate-buffered saline (PBS), was performed at 37°C for 30 minutes. The slides were mounted in Mowiol with 4-,6-Diamidino-2-phenylindole (DAPI), both purchased from Hoechst (Frankfurt am Main, Germany), and observed in a confocal laser scanning microscope (Leica TCS SP2 AOBS) with double-photon excitation. In the first step, the specimens were observed with Nomarski contrast optics. Simultaneous excitation with an argon-krypton laser at a wavelength of 488 nm for FITC and with helium-neon laser at the wavelength of 633 nm for Cy5 was used in the second step. Finally, excitation of DAPI-stained cell nuclei was performed with double-photon IR laser MIRA/VERDI at a wavelength of 795 nm. The slides incubated without primary/secondary antibodies were used as negative controls.

Mutational Analysis

Written informed consents were obtained from the parents of the patient before the genetic investigation. *ABCC2/MRP2* and *UGT1A1* genes were analyzed by direct sequencing from

genomic DNA extracted from leucocytes of peripheral blood. All exons were amplified by PCR using intronic oligonucleotide primers (Table 2).
The amplicons were gel purified, extracted with QIA quick spin columns (Qiagen, Hilden, Germany), and sequenced on an automated fluorescent DNA sequencer (AlfExpress, Amersham-Pharmacia, Uppsala, Sweden). Exons with suspected defects were cloned into a plasmid vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and wild-type and mutated alleles were sequenced separately. The presence of mutations was confirmed by PCR-restriction fragment-length polymorphism (RFLP) analysis. In addition, *ABCC2/MRP2* RNA was isolated from cultured skin fibroblasts of the patient and reverse transcribed, and fragments containing the mutations were amplified by nested PCR from cDNA and submitted to restriction analysis. Genotyping the TATAA-box of *UGT1A1* gene promoter was performed by fragment analysis of PCR products amplified from genomic DNA using Cy5-labelled forward primer. The fragments were analyzed on an automated DNA sequencer. The phenobarbital-responsive enhancer module of the *UGT1A1* gene promoter[9] was checked for the presence of the -3279T/G polymorphism by PCR-TspRI RFLP.

Results

Histology

H&E staining of the patient's liver (Figure 1A) showed normal structure, except for accumulation of brown granular pigment within the cytoplasm of hepatocytes in centrilobular zones. The pigment displayed staining properties of lipofuscin with strong PAS positivity (Figure 1B) as well as strong autofluorescence, but it did not posses the ability to reduce ammonium silver complex (negative Masson's reaction).

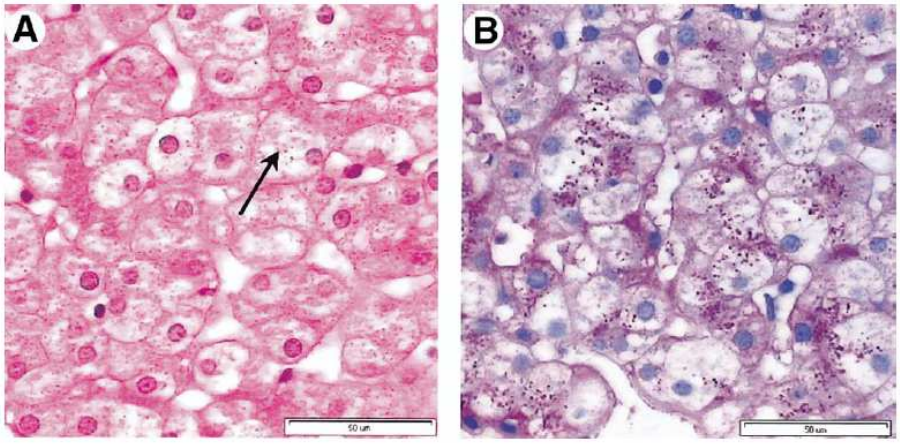

Figure 1. Liver histology and immunohistology of the index patient. H&E-stained liver tissue with brown granular deposits of lipofuscin (*A*) gave a PAS-positive reaction (*B*). The *arrow* indicates brown lipofuscin granules dispersed within the cytoplasm. (Original magnification ×400.)

Accumulation of melanin-like pigment representing the characteristic histomorphologic feature of Dubin–Johnson syndrome was therefore not observed in hepatocytes of our patient. ABCC2/MRP2 Protein Expression Immunohistologic staining showed linear positivity
for CEACAM1 in the canalicular membrane of hepatocytes in our patient, as well as in control liver tissue. In the control liver, MRP2 positivity revealed a similar plasma membrane distribution as CEACAM1, whereas detection of MRP2 in the patient's liver was totally negative. To confirm these results, confocal laser scanning microscopy (CLSM) with double immunofluorescence staining was performed. Both CEACAM1 (Figure 2A) and MRP2 (Figure 2B) colocalized on the canalicular membrane in normal liver tissue (Figure 2C). In contrast,

complete absence of MRP2 protein was observed in the liver of our patient (Figure 2E), whereas expression of CEACAM1 was not affected (Figure 2D). In addition, no MRP2 positivity was observed in any intracellular compartment of hepatocytes excluding the intracellular retention of a mutated MRP2 (Figures 2E and 2F).

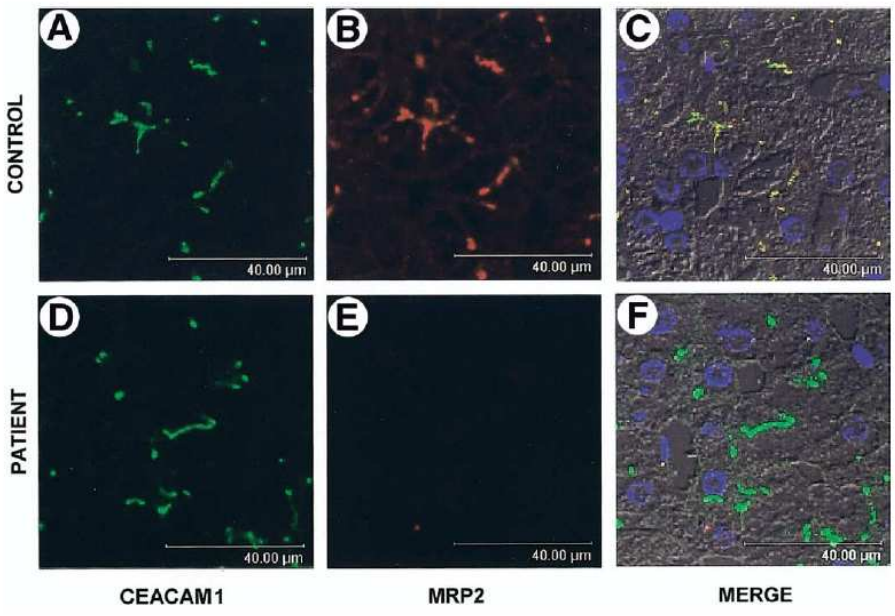

Figure 2. Confocal laser scanning micrographs with double immunofluorescence staining for CEACAM1 (*A* and *D*) and MRP2 (*B* and *E*). Cell nuclei are stained with DAPI, and cell borders are highlighted with Nomarski contrast optics (*C* and *F*). In the control liver, MRP2 and CEACAM1 colocalize with the canalicular membrane of hepatocytes (*C*, *yellow*). In contrast, in the patient's liver, MRP2 is absent on both canalicular membranes and in cytoplasm of hepatocytes (*E* and *F*). Expression of CEACAM1 is not affected (*D* and *F*). (Original magnification ×630.)

DNA Analysis

Analysis of the *ABCC2/MRP2* gene disclosed 2 novel mutations (Figure 3A): a heterozygous in-frame indel 1256insCT/delAAACAGTGAACCTGATG in exon 10 inherited from the father and a heterozygous deletion 4292delCA in exon 30 inherited from the mother. The latter mutation resulted in the frame shift and a premature stop codon at position 1461 instead of 1546. Both alleles were expressed at the RNA level in cultured skin fibroblasts. In addition, the patient appeared to be a homozygote for the A(TA)7TAA allele of the TATAA box of *UGT1A1* promoter (Figure 3B) and -3279T>G mutation in the phenobarbital-responsive enhancer module. Family analysis revealed homozygous state for both mutations in the patient's mother, whereas the father proved to be a heterozygote. No additional mutations were found in the *UGT1A1* gene.

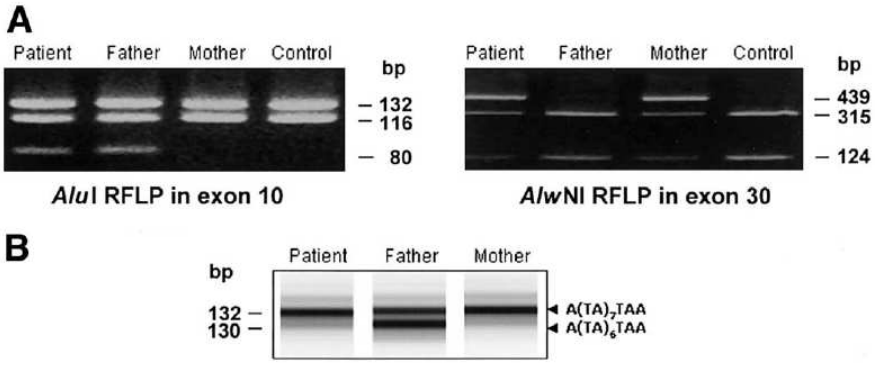

Figure 3. Heterozygous mutations detected in exon 10 and 30 of *ABCC2/MRP2* gene (*A*) and a homozygous insertion of TA in the TATAA-box of the *UGT1A1* gene promoter (*B*). The pattern of inheritance of the mutations was determined using *Alu*I RFLP (*A, left*), *Alw*NI RFLP (*A, right*), and fragment analysis (*B*), respectively.

Discussion

Clinical presentation, laboratory findings, and results of cholescintigraphy indicated the diagnosis of DJS in our patient. However, present with predominantly unconjugated hyperbilirubinemia together with the lack of the melanin-like liver pigment typically observed in subjects with DJS were not fully compatible with DJS. The degree of liver pigmentation in DJS may be variable and does not correlate with the serum bilirubin levels,[10] and even the lack of liver pigmentation has been reported in several individuals affected with inherited jaundice, whereas other family members presented with the typical pigment of DJS.[11] The pigment may not be present in early infancy,[12] although several cases of neonatal DJS with liver pigmentation have been described.[13–16] Interestingly, reexamination of the apparently pigment-free liver biopsy specimen taken during the first year of life, in a patient presenting with typical DJS in later life, revealed the presence of sporadic but typical melanin-like granules in the liver.[12] In GS, the liver tissue is histologically normal, except for the frequent presence of small amounts of stainable iron in hepatocytes.[17] Nonetheless, nonspecific accumulation of lipofuscin in centrilobular zones was reported in several cases of GS.10 An identical morphologic picture, but not with the iron deposits, was observed in our patient. Despite careful examination, we did not observe any histologic features corresponding with DJS. We believe, therefore, that prominent lipofuscin accumulation in hepatocytes, unusual in childhood, can either represent an immature form of melanin-like pigment in DJS or can be associated with the dual defect of bilirubin excretory pathway. In addition to the homozygous state for the *UGT1A1*28* allele typical for GS, our patient appeared to be a compound heterozygote for 2 novel mutations in the *ABCC2/MRP2* gene. None of the mutations impaired the transcription or stability of the corresponding messenger RNA (mRNA) as indicated by the results of *ABCC2/MRP2* cDNA analysis. The mutated protein encoded by the allele with the indel in exon 10 should be detectable with the M2III-6 antibody targeted against the epitope within the 202 C-terminal amino acids. However, the results of both conventional immunohistology and CLSM showed complete absence of MRP2 in the liver, consistent with rapid degradation of the immature protein. The second variant of the MRP2 protein truncated by 84 amino acids translated from the second allele containing the 2-bp deletion in exon 30 might escape detection with the M2III-6 antibody. Nevertheless, according to the data published recently by Nies et al,[18] incomplete forms of MRP2 protein truncated by more than 15 amino acids are not delivered to the apical membrane of hepatocytes. Absence of both mutated forms of the MRP2 protein in the liver of our patient is compatible with the diagnosis of DJS and provides a satisfactory explanation for the increased fraction of direct

reacting bilirubin found. GS manifests clinically in approximately two thirds of the homozygotes for *UGT1A1*28* allele, indicating that additional factors are required to increase bilirubin levels in the predisposed individuals. The prevalence of homozygotes and heterozygotes for *UGT1A1*28* allele among subjects affected with DJS should not differ from their prevalence in the general population. Considering the study by Shani et al[19] in which serum levels of direct reacting bilirubin were lower than 50% of total bilirubin in 15 of 101 Israeli patients with DJS, and the fact that prevalence of homozygotes for the *UGT1A1*28* allele in Israel is 13%,[20] DJS is likely one of the additional factors promoting the manifestation of the GS phenotype. Based on our experience, the noninvasive DNA analysis is the method of choice whenever the diagnosis is unclear in subjects with suspected hereditary hyperbilirubinemia, no matter the type. Indications for the liver biopsy should then be considered only if the results of genetic investigation are inconclusive.

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Abbreviations used in this paper: ABCC2, ATP-binding cassette protein C2; BSP, bromosulphthalein; CEACAM1, CEA-related adhesion molecule 1; DAPI, 4',6-diamidino-2-phenylindole; DJS, Dubin–Johnson syndrome; GS, Gilbert's syndrome; HIDA, 2,6-dimethylacetanilido-iminodiacetic acid; MRP2, multidrug resistance related protein 2; PAS, periodic acid Schiff; PCR, polymerase chain reaction; RFLP, restriction fragment-length polymorphism; UGT1A1, UDP-glucuronosyltransferase 1A1.

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The authors thank Prof. Pavel Hozak from the Department of Cell Ultrastructure and Molecular Biology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, for his kind assistance with CLSM analysis; Alexandr Rosen, Ph.D., from the Institute of Theoretical and Computational Linguistics, Faculty of Philosophy, Charles University, Prague, Czech Republic, for the translation of reference 8 from Japanese; and Prof. Martin C. Carey from Harvard Medical School, Gastroenterology Division, Brigham and Women's Hospital, and Harvard Digestive Diseases Center, Boston, Massachucetts, for his comments and help with the preparation of the manuscript.

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3.2.2 Analysis of the *ABCC2* gene in patients with Rotor syndrome

Here we hypothesised that Rotor syndrome (RS) can be an allelic variant of Dubin-Johnson syndrome (DJS), caused by the mutation in the *ABCC2* gene, and investigated *ABCC2* gene and ABCC2 protein expression in two patients with RS.

Normal expression and localisation of ABCC2/MRP2 on the canalicular membrane of hepatocytes, as assesed by confocal fluorescence microscopy, ruled out mutations abolishing the protein expression. Normal localisation of ABCC2 excluded potential defects in *RDX*, encoding radixin, a cytoskeletal protein essential in anchoring ABCC2 to the canalicular membrane (109). The immunohistologic findings, however, did not exclude mutations that can, despite normal expression of ABCC2, impair ABCC2 function. Therefore, in addition to conventional mutational screening, we performed a thorough search for less common types of mutations - exon deletions and duplications. The *ABCC2* promoter was screened to exclude a decreased expression as a result of mutations in regulatory regions of the gene and the protein coding region was sequenced from both genomic DNA and cDNA. Exonic deletions/duplications that can escape conventional analysis were tested by comparative genomic hybridisation on custom micro-arrays. No sequence alterations were found in 32 exons, adjacent intronic regions and the promoter region of *ABCC2*. We finally excluded a contribution of impaired bilirubin glucuronidation to the unconjugated fraction of hyperbilirubinemia by *UGT1A1* genotyping.

We have demonstrated, for the first time, intact expression of ABCC2/MRP2 protein in hepatocytes of Rotor syndrome patients and at the genetic level have excluded defects in the *ABCC2* gene. Thus, RS is proved to be not an allelic variant of DJS but a separate entity.

Rotor syndrome remains an enigmatic disease that promotes formation of diverse hypothesis. Traditionally, it was considered a hepatic bilirubin storage disorder (110) and several putative underlying defects were suggested, including aberrant function of glutathione-S-transferase (GST α) (111) or endoplasmic transport for conjugated bilirubin (112, 113).

Another theory deals with alternative routes for bilirubin transport across the hepatocyte *(Milan Jirsa, personal communication)*. Such routes might represent “escape” pathways like those for BA transport (see 1.2.4.). Since transport of conjugated bilirubin is often mediated together with other glucuronic conjugates, identification of the underlying defect in RS patients may have

considerable implications for the pharmacogenetics and metabolism of many endogenous and exogenous substances.

Rotor-type hyperbilirubinaemia has no defect in the canalicular bilirubin export pump.

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Liver International. 2007 May;27(4):485-91.

Abstract
Background: The cause of Rotor syndrome (RS), a rare-familial conjugated hyperbilirubinaemia with normal liver histology, is unclear. We hypothesized that RS can be an allelic variant of Dubin–Johnson syndrome, caused by mutation in *ABCC2*, and investigated *ABCC2* (gene) and ABCC2 (protein) in two patients with RS. *Methods:* A 57-year-old male presented with a 5-year history of predominantly conjugated hyperbilirubinaemia (170 mmol/l). Urinary porphyrin excretion was increased; cholescintigraphy revealed no chromoexcretion. A 68-year-old male presented with lifelong conjugated hyperbilirubinaemia (85 mmol/l). Bromosulfophthalein elimination was typical for RS. Both patients had histologically normal liver, without pigment. ABCC2 expression was investigated by confocal fluorescence microscopy. *ABCC2* was sequenced from genomic DNA and cDNA, and exon deletions/duplications were sought by comparative genomic hybridisation on a custom micro-array. *Results:* In both patients, ABCC2 was expressed unremarkably at the apical membrane of hepatocytes and no sequence alterations were found in 32 exons, adjacent intronic regions and the promoter region of *ABCC2*.
Conclusions: Rotor-type hyperbilirubinaemia is not an allelic variant of ABCC2 deficiency.

The bilirubin excretory pathway consists of two steps: conjugation of unconjugated bilirubin with glucuronic acid, catalyzed by uridine diphosphate-glucuronosyltransferase 1A1 (UGT1A1), and secretion of conjugated bilirubin into bile via ABCC2, the canalicular bilirubin export pump. Mutations in *ABCC2*, encoding ABCC2, are known to cause Dubin–Johnson syndrome (DJS, OMIM No. 237500) (1), a rare benign predominantly conjugated hyperbilirubinaemia with typical deposits of melanin-like pigment within hepatocyte lysosomes. Rotor syndrome (RS, OMIM No. 237450) represents another form of hereditary jaundice with predominantly conjugated hyperbilirubinaemia. Unlike most patients with DJS, patients with RS have no abnormal hepatic pigmentation. Total porphyrin excretion in urine is increased and the ratio of coproporphyrin isomers I:III is lower than in DJS (2). Unlike patients with DJS, patients with RS exhibit marked retention of bromosulfophthalein (BSP) after injection (3). Neither the liver nor the biliary tree is visualized by cholescintigraphy in RS (4, 5). The molecular basis of RS is unknown. The definition of RS as a pathophysiological entity distinct from DJS is based on the differences in BSP clearance and total urinary coproporphyrin output. Other features of both disorders such as liver pigmentation and visualisation of the gallbladder by cholescintigraphy are less specific (6), making the diagnosis of RS difficult to establish. We hypothesized that the phenotypic differences between RS and DJS do not exclude the possibility that RS and DJS are

allelic variants. In our study, we investigated the potential role of *ABCC2* as a candidate gene responsible for Rotor type hyperbilirubinaemia in two affected subjects.

Patients and methods

Case 1

A 57-year-old male had scleral icterus since birth. Generalized jaundice appeared for the first time at age 7 years and was diagnosed as posthepatitic. At age 18 years, he was judged unfit for compulsory military service because of jaundice. He was hospitalized, aged 52 years, owing to an acute occlusion of the central retinal artery. His total serum bilirubin concentration was 170 mmol/l, with a direct-reacting fraction of 120 mmol/l. Blood counts were within the normal ranges. Serum concentrations of aspartate aminotransferase (AST), alanine transaminase (ALT) and γ-glutamyltransferase activities and of α1-antitrypsin, copper, ceruloplasmin and bile salts were normal. No serologic evidence of infection with hepatitis A, B and C viruses or with other hepatotropic viruses was found. Urinary coproporphyrin output ranged between 80 and 500 mg/24 h (normal <200 mg/24 h); isomer I represented 57% of total coproporphyrin. Except for multiple gallstones, which were asymptomatic, no pathological changes of the liver and biliary tree were observed on ultrasonography and endoscopic retrograde cholangiopancreatography (ERCP).

In contrast, 99mTc-HIDA cholescintigraphy revealed no uptake of the radionuclide by the liver, and the bile ducts and gallbladder were not visualized (Fig. 1). No abnormality was found on microscopy of a percutaneous needle liver biopsy specimen. Jaundice has persisted, with biochemical documentation, for the subsequent 5 years, with serum concentrations of total and direct bilirubin oscillating around 170 and 120 mmol/l respectively. The family history of the patient was of potential interest because his daughter and one of his four sisters had Gilbert syndrome.

Case 2

Our second patient (7), a 68-year-old male with no family history of liver disease, had scleral jaundice since childhood. Clinical and laboratory investigations on military conscription aged 19 years revealed predominantly direct hyperbilirubinaemia with no other clinical-biochemistry evidence of hepatobiliary injury and without serologic evidence of viral hepatitis. One year later, the patient was re-examined in the Central Military Hospital (Bratislava, Slovak Republic) for fatigue, lack of appetite, weight loss, abdominal pain, dark urine and jaundice. Aside from jaundice, chronic tonsillitis and skin rash, the physical examination was unremarkable. Total serum bilirubin concentrations ranged from 41 to 121 mmol/l; direct-reacting bilirubin represented 53–72%. Total urinary coproporphyrin output was repeatedly increased, but coproporphyrin isomers were not quantitatively differentiated. Other laboratory values were within the normal ranges. BSP retention 30 min after administration of 2 mg/kg was 77.5% (normal o10%). The gallbladder was repeatedly not visualized by oral cholecystography. Exploratory laparotomy revealed a normal appearance of the liver, bile ducts and gallbladder. Normal architecture without any pigment or signs of cholestasis was found on microscopy of a biopsy specimen of liver. RS was diagnosed (7). During the subsequent 48 years, the patient's liver was assessed twice at surgery as normal in contour and color (ureterotomy for ureterolithiasis, age 44 years; cholecystectomy for gallstones, age 56 years). Persistent jaundice with predominantly conjugated hyperbilirubinaemia (total serum bilirubin ranging between 42 and 170 mmol/l with seasonal fluctuations) has been repeatedly documented.

Histology
Liver from Patient 1 was fixed in formalin; liver from Patient 2 was fixed in formalin or in Carnoy's fixative. All specimens were embedded in paraffin. Sections were stained with haematoxylin/eosin and with periodic acid–Schiff technique. Antibodies Mouse anti-ABCC2 monoclonal antibody (clone M2III-6) was purchased from Kamiya (Seattle, WA, USA). Rabbit polyclonal anti-human carcinoembryonic antigen antibody, which recognizes

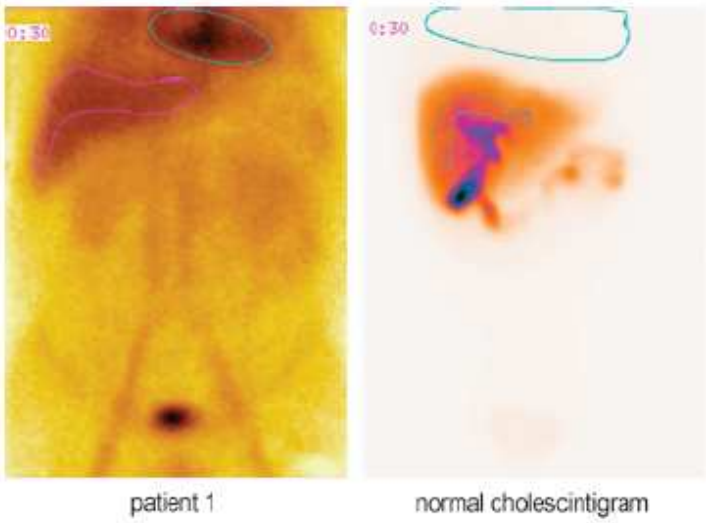

Fig. 1. Cholescintigraphy of patient 1 (left). In contrast to the normal situation (right), the radionuclide was retained in the circulation and no uptake of ^{99m}Tc-HIDA by the liver and visualization of bile ducts and gallbladder was observed.

carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1) on bile canaliculi of the human liver, was purchased from DAKO (Glostrup, Denmark) together with the EnVision Peroxidase Kit and LSAB1Kit. Fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody and Cy5-conjugated goat anti-mouse antibody were obtained from Jackson (West Grove, PA, USA).

Confocal laser scanning microscopy (CLSM)
For immunohistochemical procedures, 5 mm sections cut from formalin-fixed (Patient 1) and Carnoy's-fixed (Patient 2), paraffin-embedded tissue samples were deparaffinized and treated with 2.7% hydrogen peroxide and 0.1% sodium azide. Double immunolabelling was performed as described (8). The slides were observed in a CLSM Leica TCS SP (Leica Microsystems, Wetzlar, Germany). Simultaneous excitation with an argon–krypton laser (wavelength 488 nm) for fluorescein isothiocyanate and a helium–neon laser (wavelength 633 nm) for Cy5 was used. Sections incubated without primary antibodies were used as negative controls. Sections of a liver-biopsy specimen assessed as exhibiting minimal changes, obtained from an adult patient, were used as positive controls. Sections of a liver-biopsy specimen from a patient with proven ABCC2 deficiency (8) served as a negative control.

Mutation analysis

Written informed consents were obtained from the patients and family members before genetic investigation and skin biopsy. ABCC2 was analyzed by direct sequencing of polymerase chain reaction (PCR) products amplified from genomic DNA extracted from peripheral blood leukocytes. All exons and the 1500-bp-long promoter region were amplified by PCR using reported intronic oligonucleotide primers (8). All amplicons were gel-purified, extracted with QIA quick spin columns (Qiagen, Hilden, Germany) and sequenced on an automated fluorescent DNA sequencer (AlfExpress, Amersham-Pharmacia, Uppsala, Sweden). Analysis of copy number changes caused by exon deletions or duplications was performed by comparative hybridisation of the patient's and control male genomic DNAs. As probes, the micro-array contained PCR amplified products representing each *ABCC2* exon and 50-aminomodified 40-mer oligonucleotides corresponding to specific regions of *ABCC2* exons. Oligonucleotide sequences were designed using Oligopicker software and purchased from Illumina (San Diego, CA, USA). The PCR products (100 ng/ml) and oligonucleotides (20 mM) in 3x SSC were printed in triplicate on aminosilane-modified slides and immobilized by standard techniques that combined baking and UV cross-linking. The slides were pretreated by baking at 80 ˚C; after UV cross-linkage, they were then washed twice in 0.1% sodium dodecyl sulfate (SDS) for 2 min, twice in 0.2x SSC for 2 min and four times in MilliQ water, followed by denaturation in boiling water for 2 min. Prehybridisation was performed in prehybridisation buffer (6x SSC, 0.5% SDS, 1% bovine serum albumin). Genomic DNA was extracted by the phenol/chloroform method from peripheral blood leukocytes, fragmented with MboI restriction endonuclease (New England Biolabs, Ipswich, MA, USA) and labelled using Cy3-AP3-dUTP or Cy5-AP3-dUTP (Amersham Biosciences, Piscataway, NJ, USA). Patient samples, control samples and 5 mg of human Cot-1 (Invitrogen, Carlsbad, CA, USA) were combined and dissolved in hybridisation buffer (50% formamide, 6x SSC, 0.5% SDS, 5 x Denhardt's). Hybridisations were performed at 37 ˚C in an ArrayIt Hybridisation Cassette chamber (TeleChem International, Sunnyvale, CA, USA). Patient and control samples were analyzed in a dye swap mode with two replicates of each mode. The hybridized slides were scanned using a GenePix 4200A scanner (Axon Instrument, Union City, CA, USA) with photomultiplier gains adjusted to obtain highest-intensity unsaturated images. Data analysis was performed in the R statistical environment (version 2.2.1) using the Linear models for Microarray data package LIMMA 2.2.0, which is part of the Bioconductor project (www.bioconductor.org) (9). Raw data were processed using lowess normalisation and movingmin. Correlation between three duplicate spots per gene in each array was used to increase robustness. The linear model was fitted for each exon given from a series of arrays using lmFit function. The empirical Bayes method (10) was used to rank differential expression signal-fold changes of individual gene exons using eBayes function. *ABCC2* mRNA was isolated from cultured skin fibroblasts and subjected to reverse transcription. Overlapping ≈800 bp fragments were amplified by nested PCR from cDNA (see Table 1 for primer sequences), gel purified and sequenced. Mutations in *UGT1A1* known to be associated with Gilbert syndrome in Caucasians were detected as described (8).

Table 1. Sequences of PCR primer pairs used to amplify the overlapping fragments of *ABCC2* from cDNA

Pair number	Forward primer	Reverse primer
1	5′-TAGAAGAGTCTTCGTTCCAGACGCAG-3′	5′-AGTGCCCGCCTGGCTTTC-3′
2	5′-TTCTGAAAGGCTACAAGCGTCCTC-3′	5′-ATTGGGATTACAAGCACCATCACC-3′
3	5′-AACTTCATGCACATGCTGTGGTC-3′	5′-CCTTTATGGTGCCATTCTGAATCC-3′
4	5′-AATCCTCCTTGATATCAGCCATGC-3′	5′-TCAAGGAGTTTCTCAGGGACTTCAG-3′
5	5′-ACAGCTTTCGTCGAACACTTAGCC-3′	5′-GGATAACTGGCAAACCTGATACGG-3′
6	5′-ACCATCATCGTCATTCCTCTTGG-3′	5′-TGTTGAAAGGGTCGAGATTCATCC-3′
7	5′-ATATTGCTTCCATTGGGCTCCAC-3′	5′-TGGGTAGTAGGTTCATGGGTGTTC-3′

Results

Histology

Routine light microscopy of haematoxylin/eosinstained sections of liver from both patients found noabnormalities (Fig. 2). Pigment accumulation, the characteristic histomorphological feature of DJS, was not detected. No autofluorescence was observed on fluorescence microscopy. ABCC2 protein expression Immunohistochemical staining showed linear marking for CEACAM1 in the canalicular membrane of hepatocytes in both our patients, as well as in the positive control. CLSM with double immunofluorescence staining confirmed the localisation of ABCC2 in the canalicular membrane of hepatocytes (Fig. 3). In contrast, no ABCC2 immunostaining was observed in sections of a liver-biopsy specimen from a patient with proven ABCC2 deficiency (negative control).

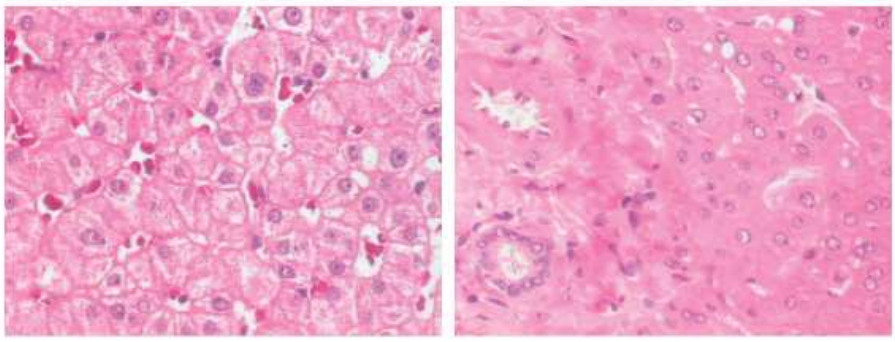

Fig. 2. Liver histology of Patient 1 (centrizonal area, needle biopsy taken at the age of 52 years) and Patient 2 (periportal area, liver excision performed in 1958 at the age of 20 years). Normal histology and cytology with no liver pigment was found in both specimens. The absence of lipopigment in hepatocytes in Patient 1 at the age of 52 is uncommon. Haematoxylin&eosin, original magnification × 400.

DNA analysis

In Patient 1, sequence analysis of *ABCC2* disclosed heterozygosity for the known synonymous polymorphism 3972C>T (I1324I, GenBank dbSNP rs3740066) in exon 28 (11) and for the polymorphism -1023G>A (GenBank dbSNP rs7910642). The sequence of cDNA corresponded with the genomic sequence. Heterozygosity for the 3972C>T polymorphism indicated that both alleles of the gene were expressed at the RNA level in cultured skin fibroblasts. A heterozygous polymorphism A(TA)7TAA of the TATAA-box and a heterozygous polymorphism -3263T/G in the phenobarbitalresponsive enhancer module were detected in the promoter region of the *UGT1A1* gene. Family analysis revealed homozygosity for both variants, A(TA)7TAA and -3263G, in the proband's daughter, indicating that both heterozygous polymorphisms are located on the same chromosome in the proband, Patient 1. Finally, Patient 1 was homozygous for the wild-type 211G allele. Sequence analysis of *ABCC2* in Patient 2 revealed homozygosity for two

known polymorphisms: 3972C>T (I1324I) in the protein-coding region and -24C>T (GenBank dbSNP rs717620) in the 50-untranslated region (11). As in Patient 1, the sequence of cDNA corresponded with the genomic sequence. In contrast to Patient 1, the expression of individual alleles could not be addressed because no heterozygous sequence variation was present in the transcribed mRNA. The patient was found to be homozygous for the wild-typealleles A(TA)6TAA and -3263T of the *UGT1A1* gene promoter and for the wild-type 211G allele in the first exon of *UGT1A1*. Comparative genomic hybridisation to a custom micro-array revealed no significant copy number changes in any of 32 exons of *ABCC2* in either Patient 1 or 2.

Discussion

We attempted to investigate the role of *ABCC2* in two subjects with Rotor-type hyperbilirubinaemia; to our knowledge, ours is the first such attempt. Normal expression and localisation of ABCC2 on the canalicular membrane of hepatocytes ruled out the mostcommon findings seen in DJS, when mutations in both alleles of *ABCC2* abolish ABCC2 expression.

Normal localisation of ABCC2 excluded potential defects in *RDX*, encoding radixin, a cytoskeletal protein essential in anchoring ABCC2 to the canalicular membrane (12). The immunohistologic findings, however, did not exclude two possibilities. Firstly, a mutation-impairing function but not expression of ABCC2 might be present in at least one allele of *ABCC2*. Secondly, a mutation in the regulatory region of *ABCC2* might decrease but not abolish ABCC2 expression. Both possibilities were checked by sequence analysis of the coding and promoter regions of *ABCC2*. In addition to conventional mutational screening, a thorough search for less common types of mutations – exon deletions and duplications – was performed; none was found. Finally, a contribution of Gilbert syndrome to the unconjugated fraction of elevated serum bilirubin was excluded by *UGT1A1* genotyping.

The rationale for investigation of *ABCC2* and ABCC2 in RS, which is considered a disorder of hepatic bilirubin storage (13), can be questioned. Significant reduction in the actual or apparent capacity of hepatocytes to store unconjugated bilirubin, unconjugated BSP and indocyanine green (which does not undergo conjugation) can result from decreased concentrations of 'ligandin' in cytosol of hepatocytes; from decreased hepatocellular uptake of unconjugated bilirubin, BSP and indocyanine green; and from occupation of binding sites of intracellular 'ligandin' – proteins belonging mainly to the a-class of the glutathione-S-transferase family (GST-α). The hypothesis that RS is a primary disorder of hepatic bilirubin storage is supported by the kinetics of selected anionic dyes and by the immunohistological findings of Abei M et al. (14). To the best of our knowledge, these findings have not been confirmed by quantitative analysis of GST-α isoenzymes or by mutational analysis of the corresponding *GSTA1-5* genes. A compensatory upregulation of GST-α family proteins in the liver has been detected in Gsta4 null mice; no jaundice has been observed in these animals (15). If the same is true for single gene defects in *GSTA1* or *GSTA2* (less likely in *GSTA3* and *GSTA5* because their expression is low), such defects cannot be expected to reduce substantially the total concentration of GST-α family proteins in the liver.

Defective uptake of unconjugated bilirubin and other organic anions is not compatible with predominantly conjugated hyperbilirubinaemia in RS. Thirdly, reduction of hepatic bilirubin storage capacity can be caused by occupation of the binding sites of 'ligandin'. Retention of 'ligandin'-binding substrates in cytosol may arise from changes in affinity of mutated *ABCC2* for a subgroup of ABCC2 substrates.

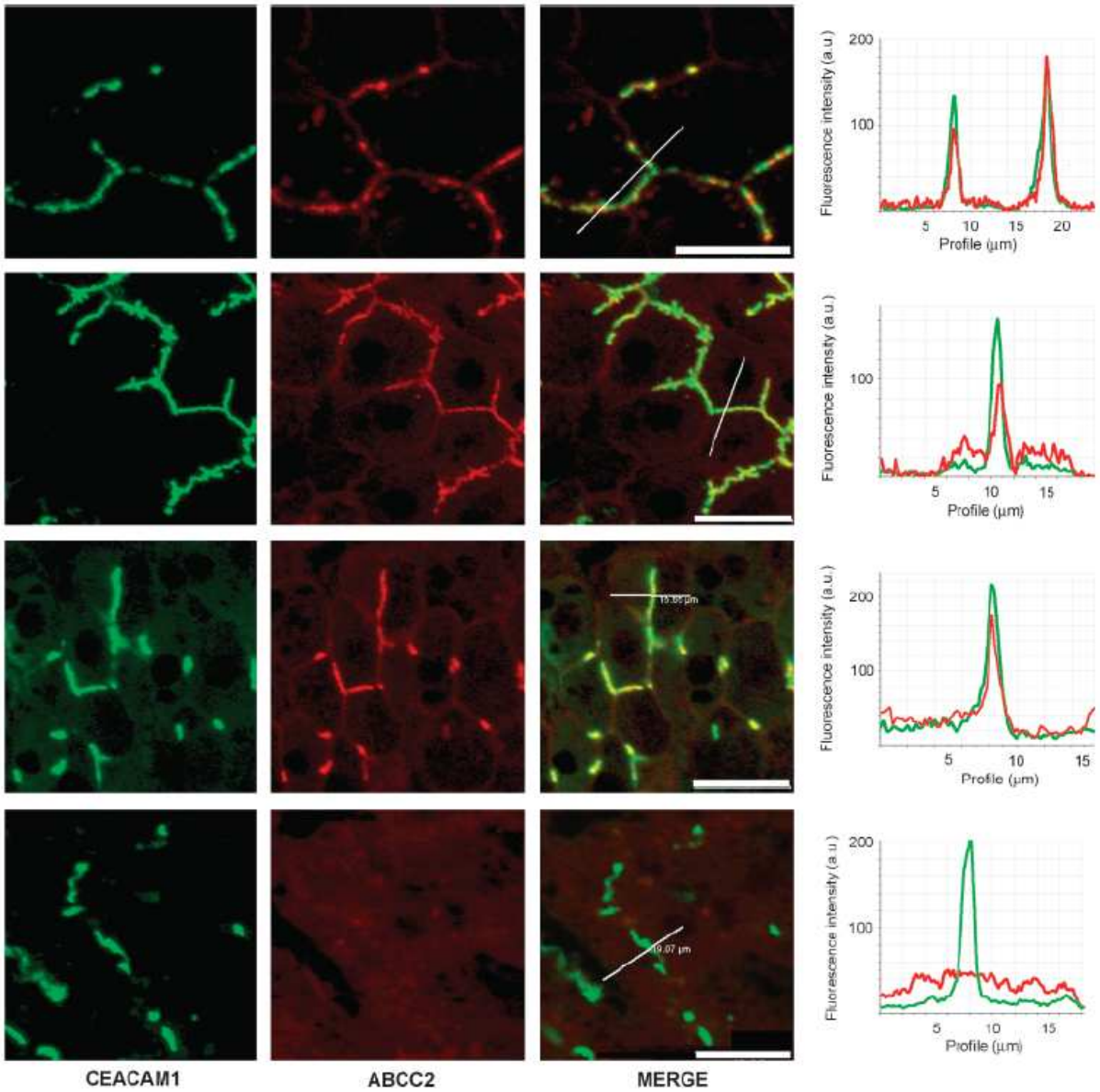

Fig. 3. Confocal laser scanning micrographs with double immunofluorescence staining for CEACAM1 (left column) and ABCC2 (middle column). In the control liver as well as in both samples from the patients with RS, ABCC2 and CEACAM1 colocalize with the canalicular membrane of hepatocytes (right column – yellow color, and graphs). In the liver of a patient with proven ABCC2 deficiency (bottom slides), ABCC2 protein is absent both at canalicular membranes and in the cytoplasm of hepatocytes, whereas expression of CEACAM1 is not affected. Bar = 20 μm, original magnification × 400.

ABCC2 can be mutated in such a way that retained nonbilirubin substrates do not upregulate expression of ABCC3 in compensation for impaired function of ABCC2.

Mutations affecting the affinity of ABCC2 for various substrates have been documented by Ito et al. (16–18). Our presented results exclude this possibility in these patients. Alternatively, 'ligandin'-binding substrates may be retained in cytosol of hepatocytes owing to deficient transport of polar conjugates from the endoplasmic reticulum (ER) to the cytoplasm. Conjugation takes place on the luminal aspect of ER (19, 20). Transport of highly polar

conjugates is likely mediated by an ATP-independent permease specific for conjugates of bilirubin (and possibly other substrates) with glucuronic acid (19, 21). Production of bilirubin glucuronides in the ER lumen and activity of the canalicular bilirubin export pump ABCC2, which keeps cytoplasmic concentrations of bilirubin glucuronides low, constitute the driving force for translocation of conjugated bilirubin across the ER membrane. In the case of impaired export, conjugated bilirubin should be retained in the ER lumen and secreted into the plasma via exocytosis. Increased intra-ER concentrations of conjugated bilirubin (and perhaps other glucuronides) may decrease the rates of conjugation of the corresponding substrates. Unconjugated substrates retained in cytosol (owing to deficient export of conjugates from the ER to the cytoplasm) bind to GST-a and decrease hepatic storage capacity for unconjugated bilirubin, unconjugated BSP and indocyanine green as well as the transport maximum for BSP.
In conclusion, we have shown that RS is not an allelic variant of DJS. The discovery of the molecular background of RS would be helpful in differentiating among forms of hereditary conjugated jaundice.

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4. General discussion and future perspectives

Cholestasis is a leading manifestation of numerous distinct hepatobiliary disorders. Most hereditary intrahepatic diseases present as a syndrome of neonatal cholestasis (NCS). Rather uniform clinical and laboratory findings together with peculiarities of the neonatal and early infant periods (all infants have a certain degree of neonatal cholestasis on a physiological basis) hamper straightforward diagnostic assessment.

Rapid diagnosis is essential for prevention of permanent liver damage. Traditionally, the diagnosis was based on clinical, laboratory, imaging and histological investigation. However, such methods are unable to distinguish the variety of underlying triggering defects that often lead to a uniform clinical and/or histopathological picture. It was progress in genetics and molecular biology that enabled better understanding of the molecular mechanisms of hepatobiliary disorders and resulted in improvement of diagnostic accuracy in this area.

More than ten years ago, in 1998, genetic definition began for a heterogeneous group of the disorders with similar clinical presentations and – unusual in conditions that manifest conjugated hyperbilirubinemia – normal range or low levels of γGT. Disruption of the *ATP8B1* (FIC1) and the *ABCB11* (BSEP) genes was proved to cause many instances of low γGT cholestasis with variable phenotypes that range from progressive early-onset to remitting and late-onset forms (47, 89). In the same year, two patients with mutations in the *ABCB4* (MDR3) gene, disruption of which results in high γGT cholestasis, were characterised (114).

Further identification of genes encoding hepatocellular transporters and understanding of function of the encoded proteins enabled a rapid diagnosis of patients with various hereditary cholestatic liver diseases. At the same time, it prompted detailed sequence analysis and characterisation of disease-associated mutations, as well as of the impact of such genetic modifications on protein structure and function. In parallel, the studies of nuclear hormone receptors and their role in the control of transcription uncovered the complexity of these genes's regulation under both, physiological and pathological conditions. Recent achievements also stimulated interest in mutation-tailored therapy, in order to modulate disease phenotypes resulting from specific genetic defects.

Our work has contributed to the current knowledge of hepatobiliary defects by characterisation of mutations and their phenotypic consequences in PFIC1 (*ATP8B1* deficiency), PFIC2 (*ABCB11* deficiency) and Dubin-Johnson syndrome (*ABCC2* deficiency), identification and

functional analysis of the previously unknown regulatory regions of the *ATP8B1* gene, description of a yet unrecognised mixed type digenic hyperbilirubinemia and by exclusion of a putative *ABCC2* genetic defect in Rotor syndrome. Moreover, the fact that we have implemented molecular diagnostics of low γGT cholestases and hereditary jaundice in the Czech Republic has also a practical significance: At present, 38 *ABCB11*, 13 *ATP8B1* and 5 *ABCC2* deficient patients have been diagnosed in Laboratory of Experimental Hepatology, IKEM, Prague.

Detailed characterisation of an underlying genetic defect and its impact on mRNA and/or protein levels in different tissues are the essential prerequisites for the future progress in therapy of hereditary diseases. Mutation-specific therapy, an alternative tool to a gene-replacement method, is aimed to attenuate phenotypic consequences of a specific genetic defect using various genetic modifiers including small chemical chaperons and splicing factors. For example, in patients carrying a mutation that affects splicing but with partially preserved protein expression, both aberrantly and correctly spliced transcripts are generated (115). The ratio of generated transcripts fluctuates among the patients and, interestingly, among different organs of the same patient. The resulting variable phenotype can be modulated by splicing factors able to increase levels of correctly spliced transcript.

In vitro experiments demonstrated successful modulation of mRNA defective splicing or mutant protein processing of *ABCB11*, *ATP8B1*, *ATP7B* and *CFTR* genes (93, 116-121). Byrne et al. (93) showed partial restoration of wild-type splicing for two *ABCB11* mutations after exogenous addition of splicing factor SC35. Similarly, Nissim-Rafinia et al. (117) demonstrated increases in correctly spliced *CFTR* transcripts after overexpression of splicing factors Htra2-β1 and SC35, followed by restoration of *CFTR* protein function.

The effects of different *ATP8B1*, *ABCB11*, *ATP7B* and *CFTR* mutants resulting in protein misfolding and/or mislocalisation were ameliorated after addition of 4-phenylbutyrate (4-PBA) (116, 118, 119). Although the exact mechanism of 4-PBA action is not fully understood, this orally administrable histone deacetylase inhibitor functions as a small molecule chaperone able to increase the cell surface expression of a number of multispanning membrane proteins.

The above mentioned strategies indeed do not “treat” the genetic defects, but represent a promising tool potentially capable to ameliorate the disease phenotype by modulating the local environment.

After a period of extensive molecular discoveries, current effort on the field of hereditary liver diseases is focused primarily on:

i) The identification of novel candidate loci involved in cholestatic disorders including malformations determined by mutations in several different genes.

ii) The characterisation of *cis*- and *trans*-acting elements involved in the regulation of gene expression (e.g. non-coding regions of the genes, nuclear receptors, a number of different small RNA species).

iii) The correlation of genotype with phenotype, and the prediction of impact of individual mutations on the severity of the disease.

iv) The extensive characterisation of the protein function for hepatocyte transporters.

Hereditary liver diseases constitute a large family of manifestations with particularly complex genetic and phenotypic backgrounds. Therefore, in addition to the characterisation of genes and their mutations potentially leading to malformations, it is essential to examine the processes of gene transcriptional and post-transcriptional regulation as well as protein targeting (localisation) and function. Only detailed understanding of the molecular mechanisms responsible for the particular phenotype will allow us to design highly specific treatment, small molecule-based or gene therapy, for patients with hereditary liver diseases.

5. **Summary**

1. We identified novel mutations associated with Dubin-Johnson syndrome, Progressive familial intrahepatic cholestasis type 1, (FIC1 deficiency) and Progressive familial intrahepatic cholestasis type 2 (BSEP deficiency) manifesting with both progressive early-onset and mild recurrent phenotypes.
2. We showed that severe *ABCB11* deficiency confers a risk of hepatobiliary malignancy particularly for those carrying 2 null mutations. Close surveillance of PFIC2 patients retaining their native liver is thus essential.
3. We proved that BSEP immunostaining is a powerful tool in diagnostic assessment of patients with severe hepatobiliary disease in which *ABCB11* deficiency is suspected, since immunohistochemically detectable BSEP is typically absent, or much reduced in severe hepatobiliary disease owing to *ABCB11* mutations.
4. We provided fundamental data on the complexity of the *ATP8B1* gene regulation, identifying new *ATP8B1* mRNA isoforms that differ in 5'UTR and in both, transcriptional and translational efficiency. The basal expression of the *ATP8B1* gene in liver and intestine is driven by a promoter with house-keeping like properties and is not regulated by farnesoid X receptor and bile acids.
5. We characterised a novel type of digenic mixed hyperbilirubinemia - a distinct type of constitutive jaundice resulting from co-inherited defects in the *ABCC2* and *UGT1A1* genes.
6. We demonstrated that Rotor syndrome is a disease distinct from Dubin-Johnson syndrome and not its allelic variant.

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8. List of author's publications, presentations and awards

8.1 Publications

1. *Strautnieks SS, Byrne JA, Pawlikowska L., Cebecauerová D. et al.*
   Severe bile salt export pump deficiency: 82 different *ABCB11* mutations in 109 families. Gastroenterology. 2008 Apr;134(4):1203-14. Jan 18.
   IF=12.591

2. *Hrebicek M, Jirasek T, Hartmannova H, Noskova L, Stranecky V, Ivanek R, Kmoch S, Cebecauerova D et al.*
   Rotor-type hyperbilirubinaemia has no defect in the canalicular bilirubin export pump. Liver Int. 2007 May;27(4):485-91.
   IF=2.559

3. *Kotalová R., Cebecauerová D., Knisely A.S., Hřebíček M., Jirsa M.*
   Progressive Familial Intrahepatic Cholestasis – Manifestations and Diagnosis in Infancy. Čes.-slov.Pediat., 2006, roč.61, č.4, s.200-206

4. *Cebecauerova D., Jirasek T., Budisova L., Mandys V., Volf V., Novotna Z., Subhanova I., Hrebicek M., Elleder M., Jirsa M.*
   Dual hereditary jaundice: simultaneous occurrence of mutations causing Gilbert's and Dubin-Johnson syndrome.
   Gastroenterology. 2005 Jul;129(1):315-20.
   IF=12.386

5. *Jirsa M., Cebecauerová D., Budišová L., Chuzhanova N., Hřebíček M. et al.*
   Indel in the *FIC1/ATP8B1* gene-a novel rare type of mutation associated with benign recurrent intrahepatic cholestasis.
   Hepatol Res. 2004 Sep;30(1):1-3.
   IF=1.766

8.2 Conference Presentations related to thesis

2003 CEBECAUEROVÁ, Dita, et al. Cholestatické nemoci jater s normální hladinou sérového cholesterolu. *VII.kongres o ateroskleróze*, Špindlerův Mlýn, 4. - 6. 12. 2003. **Poster.** Abstract in: *Diabetologie, metabolismus, endokrinologie a výživa*, 2003, vol. 6, suppl. 3, p. 37.

2004 CEBECAUEROVÁ, Dita, et al. Familial predominantly unconjugated hyperbilirubinemia caused by a coincidence of two inborn errors of bilirubin metabolism: Dubin-Johnson syndrome and Gilbert syndrome. *5th Biennial Alex Mowat Meeting Paediatric Hepatology Update Young Investigator´s Forum*, London, UK, 5. 4. 2004. **Presentation.**

CEBECAUEROVÁ, Dita, et al. Familiární převážně nekonjugovaná hyperbilirubinémie způsobená kombinací Dubin-Johnsonova a Gilbertova syndromu. *XXXII. Májové hepatologické dny*, Karlovy Vary, 12.-14. 5. 2004. **Presentation.**

CEBECAUEROVÁ, Dita, et al. Atypický fenotyp Dubin-Johnsonova syndromu vyvolaný mutacemi ve dvou klíčových genech exkreční dráhy bilirubinu. *VI. český pediatrický kongres*, Ostrava, 8.-11. 9. 2004. **Poster.**

JIRSA, Milan, **CEBECAUEROVÁ, Dita,** et al. Indel in the *FIC1*/*ATP8B1* gene – a novel rare type of mutation associated with benign recurrent intrahepatic cholestasis. *12th United European Gastroenterology Week*, Praha, 25. - 29. 9. 2004. **Poster.** Abstract in: *Gut*, 2004, vol. 53, suppl. VI, p. A89.

CEBECAUEROVÁ, Dita, et al. Atypical phenotype of Dubin-Johnson syndrome caused by co-inherited defects in two crucial genes of bilirubin excretory pathway: ABCC2/MRP2 and UGT1A1. *12th United European Gastroenterology Week*, Praha, 25. - 29. 9. 2004. **Poster. A**bstract in: *Gut*, 2004, vol. 53, suppl. VI, p. A90.

CEBECAUEROVÁ, Dita, et al. Benigní rekurentní intrahepatální cholestáza podmíněná mutacemi v genu pro kanalikulární přenašeč žlučových kyselin (BSEP). *Podzimní pracovní de, České hepatologické společnosti*, Olomouc, 25. 11. 2004. **Presentation.**

2005 CEBECAUEROVÁ, Dita, et al. Genetická heterogenita benigní rekurentní intrahepatální cholestázy. *XXXIII. Májové hepatologické dny 2005*, Karlovy Vary, 4. -6. 5. 2005. **Presentation.**

JIRÁSEK, Tomáš, **CEBECAUEROVÁ, Dita**, et al. Duální vrozená žloutenka podmíněná mutacemi ve dvou klíčových genech exkreční dráhy bilirubinu. *13. sjezd českých a slovenských patologů s mezinárodní účastí*, Rožnov pod Radhoštěm, 14 .-16. 9. 2005. **Presentation.**

KOTALOVÁ, Radana, **CEBECAUEROVÁ, Dita**, et al. Sklerozující cholangitida jako první projev vrozeného deficitu žlučových fosfolipidů. *17. pracovní dny dětské gastroenterologie a výživy*, Hradec Králové, 4.-5.11.2005. **Presentation.**

2007 CEBECAUEROVÁ, Dita, et al. Characterisation of the non-coding region of the *ATP8B1* gene. *42nd Annual Meeting of the European Association For The Study Of The Liver*, Barcelóna, Spain, 11. - 15. 4. 2007. **Presentation.**

2008 CEBECAUEROVÁ, Dita, et al. Characterisation of the non-coding region of the *ATP8B1* gene. 7th *Biennial Alex Mowat Meeting Paediatric Hepatology Update Young Investigator´s Forum,* London, UK, April, 2008. **Presentation.**

8.3 Conference Presentations not related to thesis (selection)

2007 CAREY, Ivana, MYTILINAIOU, Maria, GIANNATTASIO, Antonietta, BANSAL, Sanjay, CHEESMAN, Paul, **CEBECAUEROVA,** Dita, MIELI-VERGANI, Giorgina, VERGANI, Diego. HBeAg seroconversion correlates with a high number of core gene mutations and with genotypes B and D in paediatric patients. *58th Annual Meeting of the American Association for the Study of Liver Disease, November 2-6, 2007, Boston,* Abstract in Hepatology, Vol. 46, No. 4, Suppl. 1, 2007. **Poster.**

2008 CAREY, Ivana, **CEBECAUEROVA, Dita** et al. Response to pegylated interferon/ribavirin in chronic hepatitis C in children is predicted by pre-treatment number of acivated natural killer (NK) cells. *59th Annual Meeting of the American Association for the Study of Liver Disease,* October 31 - November 4, 2008, San Francisco, Abstract in Hepatology Vol.48, No. 4 (Suppl.) **Poster.**

2009 CAREY, Ivana, MENDES, Andrea, BANSAL, Sanjay, SUBRAMANIAM, Pushpa, LONGHI, Maria Serena, **CEBECAUEROVA,** Dita, MIELI-VERGANI, Giorgina, VERGANI, Diego. Sharp decrease in HCV-specific interferon-γ and IL-10 production during antiviral therapy with pegylated interferon and ribavirin predict sustained virological response in children with chronic hepatits C. *60th Annual Meeting American Association for the Study of Liver Disease, October 30 - November 3, 2009, Boston.* Abstract in Hepatology Vol.50, Suppl.4. **Poster.**

8.4 Awards

2008 Second Prize for abstract and presentation, 7th Biennial Alex Mowat Meeting, Young Investigators' Forum, 5th April 2008, London, Title: Characterisation of the non-coding region of the *ATP8B1* gene

2005 EASL Sheila Sherlock Fellowship 2005, Hosting Institution: Institute of Liver Studies, King's College Hospital, London, Project Title: Characterisation of the non -coding region of the *ATP8B1* gene

2005 Cena ČHS a ČGS za nejlepší publikaci za rok 2005: Cebecauerova D., Jirasek T., Budisova L., Mandys V., Volf V., Novotna Z., Subhanova I., Hrebicek M., Elleder M.,

Jirsa M. Dual hereditary jaundice: simultaneous occurrence of mutations causing Gilbert's and Dubin-Johnson syndrome. Gastroenterology. 2005 Jul;129(1):315-20.

9. Abbreviations

ABCB11 – ATP-binding cassette protein class B, type 11

ABCB4 – ATP-binding cassette protein class B, type 4

ABCC2 – ATP-binding cassette protein class C, type 2

ABCG5/ABCG8 – ATP-binding cassette protein, class G, type 5/8

AE2 – Chlorid-bicarbonate anion exchanger 2

AGS – Alagille syndrome

AMA – Antimitochondrial antibodies

ARC – Arthrogryposis, renal dysfunction and cholestasis syndrome

ATP8B1 – ATPase class I., type 8B, member 1

ASBT – Apical sodium-dependent bile salt transporter

tASBT – Truncated ASBT

BAAT – Bile acid coenzyme A - amino acid N-acetyltransferase

BRIC – Benign recurrent intrahepatic cholestasis

BS – Bile salts

BSEP – Bile salt export pump

CA – Cholic acid

CDCA – Chenodeoxycholic acid

CEA – Carcinoembryonic antigen

CEACAM1 – CEA cell adhesion molecule 1

CFTR – Cystic fibrosis transmembrane conductance regulator

CIC – Contraceptive-induced Cholestasis

CIRH1A – Cirhin 1A

CLDN1 – Claudin 1

CLSM – Confocal laser scanning microscopy

DAPI – 4',6-diamidino-2-phenylindol

DCA – Deoxycholic acids

DJS – Dubin-Johnson syndrome

EPHX1 – Microsomal epoxide hydrolase

FAB-MS – Fast atom bombardment – mass spectroscopy

FCS – Fetal calf serum

FHCA – Familial hypercholanemia

FIC1 – Familial intrahepatic cholestasis 1 protein

FITC – Fluorescein isothiocyanate

FXR – Farnesoid X-activated receptor

GC-MS – Gas chromatography-mass spectroscopy

GGT – see γGT below

GS – Gilbert syndrome

HMG-CoA reductase – Hydroxy-methyl-glutaryl coenzyme A reductase

gtPBREM – Glucuronosyltransferase phenobarbital response enhancing motif

IBABP – Ileal bile acid-binding protein

ICP – Idiopathic cholestasis of pregnancy

ILVASC – Ichthyosis, Leukocyte vacuoles, Alopecia, Sclerosing Cholangitis

IVS – Intronic sequence variation

JAG1 – Jagged-1

LCA – litocholic acid

LPAC – Low phospholipid associated cholelithiasis

LpX – Lipoprotein X

Mdr2 – Murine Multidrug resistance protein 2

MDR3 – Multidrug resistance protein 3

MLPA – Multiplex ligation-dependent probe amplification

MRP4 – Multidrug resistance protein 4

MRP2 – Multidrug resistance-associated protein 2

NAIC – North American Indian childhood cirrhosis

NTCP – Sodium/taurocholate cotransporting polypeptide

OATP – Organic anion transporter

OSTα/β – Organic solute transporter α/β

OMIM – Electronic database Online Mendelian Inheritance in ManTM

PAS – Periodic acid – Schiff

PBD – Partial biliary diversion

PCR – Polymerase chain reaction

PFIC – Progressive intrahepatic familial cholestasis

RFLP – Restriction fragment length polymorphism

RS – Rotor syndrome

RXR – Retinoid X receptor

SNP – Single nucleotide polymorphism

SHP – Small heterodimer partner

TBS – Tris buffered saline solution

TCA – Taurocholic acid

TJP2 – Tight junction protein 2

TMEM50B – Homo sapiens transmembrane protein 50B

UDCA – Ursodeoxycholic acid

UGT1A1 – Uridine diphosphate glucuronosyltransferase 1A1

VPS33B – Homolog of yeast vacuolar protein sorting 33B

γGT – Gamma-glutamyltransferase

4-PBA – 4-phenylbutyrate