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PhD Thesis – Short report

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

Dita Cebecauerová

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PhD program: Biochemistry and pathobiochemistry

Program committee chairman: Prof. MUDr. Jiří Kraml, DrSc.

Supervising department: Institute for Clinical and Experimental Medicine, Laboratory of Experimental Hepatology

Supervisor: MUDr. et Mgr. Milan Jirsa, CSc., associate professor

Opponents: Prof. MUDr. Radim Brdička, DrSc.
Prof. MUDr. Macek Milan Jr., DrSc.

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Contents

Summary (English)

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.

Chapters related to low γGT cholestasis

- characterise rare type of mutation associated with benign course of PFIC type I (formerly BRIC1) and explain the putative mechanisms of mutation origin.
- provide extensive study of severe forms of *ABCB11* deficiency (PFIC2) including genotype-phenotype correlations in 109 affected families, evaluation of the specific *ABCB11* genotypes' impact on BSEP immunostaining and risk of hepatobiliary malignancy.
- identify and characterise yet unknown regulatory regions of *ATP8B1*, a gene mutated in Progressive Familial Intrahepatic Cholestasis type I. The studies demonstrate the complex structure of *ATP8B1* gene, identify novel untranslated exons and three independent promoter regions and provide a functional study of main *ATP8B1* promoter.

Chapters related to hereditary jaundice

- describe novel type of mixed, predominantly unconjugated hyperbilirubinemia that was proved on molecular level as digenic disorder caused by mutations in *ABCC2* and *UGT1A1* genes. The importance of the genetic analysis is illustrated in this case by misleading clinical presentation in the patient, who was at first reported as Dubin-Johnson syndrome, despite the lack of typical DJS liver pigmentation and predominantly unconjugated type of jaundice.
- hypothesise the role of *ABCC2*, the gene mutated in Dubin-Johnson syndrome, in another hereditary conjugated jaundice: Rotor syndrome. Present study demonstrates for the first time intact expression of ABCC2/MRP2 protein in hepatocytes of Rotor syndrome patients and on the genetic level excludes defects of *ABCC2* gene.

Souhrn (Česky)

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*.

Oddíly týkající se cholestáz s nízkou hladinou gammaglutamyltransferázy
- popisují vzácný nalezený typ inserčně-deleční mutace asociované s benigním průběhem progresivní familiární intrahepatální cholestázy 1. typu a diskutují možný mechanismus vzniku mutace a její pravděpodobný vliv na funkci proteinu FIC1.
- shrnují rozsáhlou genetickou analýzu pacientů se závažnou formou progresivní intrahepatální cholestázy 2. typu, podmíněnou mutacemi v BSEP (Bile Salt Export Pump). Studie se zabývá korelací genotypu a fenotypu u 109 postižených rodin, diskutuje vliv jednotlivých mutací, zejména typ, lokalizaci a stupeň konzervace, na imunohistologickou detekci proteinu a tíži klinických projevů, včetně rizika maligního zvratu.
- se zabývají identifikací a charaterizací dosud nepopsaných regulačních oblastí genu *ATP8B1* mutovaného u progresivní familiární intrahepatální cholestázy 1. typu (PFIC1) a představují první funkční studii demonstrující nezávislost mezi transkripční regulací genu a stimulací žlučovými kyselinami.

Oddíly týkající se dědičně podmíněných žloutenek
- definují nový typ smíšené převážně nekonjugované žloutenky, u níž byl na základě molekulárního vyšetření potvrzen digenní původ podmíněný mutacemi v genech *ABCC2* a *UGT1A1*. Gen *ABCC2* kóduje protein ABCC2/MRP2 (Multidrug resistance-associated protein 2), který v kanalikulární membráně hepatocytů funguje jako přenašeč organických aniontů včetně konjugovaného bilirubinu. Gen *UGT1A1* kóduje UDP-glukuronosyltransferázu zodpovědnou za konjugaci bilirubinu v játrech. Mutace v těchto genech podmiňují Dubin-Johnsonův respektive Gilbertův syndrome. U studovaného probanda, nositele mutací v obou těchto genech, došlo k překryvu fenotypu obou onemocnění a tím k zavádějícímu obrazu smíšené žloutenky. Až genetické vyšetření odhalilo podstatu choroby a pomohlo objasnit atypický klinický obraz.

- diskutují možnou roli genu *ABCC2* u pacientů s Rotorovým syndromem (RS), který se od Dubin-Johnsonova syndromu (DJS) liší i přes podobný klinický obraz v některých laboratorních a histologických nálezech.

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

Material and methods

1) Mutational analysis
(Related to studies of the ABCC2, UGT1A1, ABCB11 and ATP8B1 genes)

Written informed consents were obtained from the patients and/or parents of the patients before the genetic investigation. 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. 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) or using ABI Big Dye (Version 3.1) on 3100 automated DNA Sequencer (Applied Biosystems, Foster City, USA). Exons with suspected defects were cloned using pCR2.1-TOPO or Zero-blunt kit (Invitrogen, Carlsbad, CA) or pDrive Cloning Vector (Qiagen, Hilden, Germany). Wild-type and mutated alleles were sequenced separately. The presence of mutations was confirmed by RFLP analysis.

2) 5' Rapid Amplification of cDNA Ends, 5'RACE
(Related to the ATP8B1 gene study)

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 5×10^6 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.

3) Quantitative Real–time PCR, qRT-PCR
(Related to the ATP8B1 gene study)

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 *ATP8B1* untranslated exons. 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. One hundred 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). One 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 *ATP8B1* transcripts and the non-variable calibrator 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 Microsoft Excel.

4) Plasmid construction
(Related to the ATP8B1 gene study)

Twelve fragments of the 5´UTR of the *ATP8B1* gene were PCR amplified using human genomic DNA as a template, Pfx DNA polymerase (Invitrogene) and specific primers with *Xho*I restriction sites. PCR product was cloned (Invitrogen Zero blunt kit or Qiagen Cloning kit), sequenced, digested with *Xho*I and ligated into *Xho*I 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.

5) Cell culture and transfection
(Related to the ATP8B1 gene study)

HepG2 cell lines were 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 LucATP8B1 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.

6) Reporter gene assay
(Related to the ATP8B1 gene study)

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 normalised to the *Renilla* luciferase and pGL3-Basic activities.

7) Immunohistochemistry
(Related to the ABCC2 and ABCB11 genes studies)

The *ABCC2* gene study:
For immunohistochemical procedures, 5-µm-thick sections cut from formalin-fixed, paraffin-embedded tissue samples were deparaffinised 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.

The *ABCB11* gene study:
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 (1). 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,UK). Findings were evaluated by light microscopy as described (2, 3).

8) Confocal laser scanning microscopy.
(Related to the ABCC2 gene study)

Microsections were deparaffinised 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

Results and Discussion

1. Low gamma-glutamyl cholestasis

1.1. Mutational analysis of the *ATP8B1* and *ABCB11* genes in Czech patients with mild phenotype (formerly Benign recurrent intrahepatic cholestasis – BRIC)

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 (4). 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") (5) and the prediction of phenotype severity according to the underlying defect appears to be limited (6). 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 (7). 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 (4, 8, 9). 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* (6).

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 (9). 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 (10).

The p.Ile661Thr mutation in heterozygous form together with nonsense mutations was found in progressive forms of ATP8B1/FIC1 deficiency (6). Thus the heterozygous state is associated with either episodic or chronic progressive cholestasis.

1.2. Mutational analysis of the *ABCB11* gene in Czech patients with severe phenotype (Progressive familial intrahepatic cholestasis type 2 - PFIC2)

The mutation p.Asp482Gly, identified in proband P1, is a known pathogenic defect associated in homozygous form with PFIC2 (7, 11). The mutation is located within a highly conserved nucleotide binding fold (NBF) 1 domain. Expression of BSEP varies significantly for this mutation (11).

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 (12). 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 (2, 3, 11). p.Asp482Gly mutation appears to confer a particular risk as 16% of BSEP-deficient patients who

developed hepatocellular or cholangiocellular carcinoma carried this mutation (12).

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. (12) 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. (13) 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. (14). In vitro expression demonstrated no protein production for p.Val284Leu (12), 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 (13, 15-20).

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 correlation 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. (6) 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. (2), 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 (21).

1.4. Characterisation of the regulatory regions of the *ATP8B1* gene

Despite the fact that the *ATP8B1* gene was identified in 1998 (4), 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 of Klomp et al. (6) *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 (11). 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 (22).

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 (4, 23, 24). Our results thus

support uniform, bile acid-independent mechanisms regulating *ATP8B1* basal expression and function across different tissues.

2. Hereditary jaundice

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 (25). Rarely the defective protein is expressed and available for immunohistological detection (26).

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 (27).

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 (28). 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 (29) and several putative underlying defects were suggested, including aberrant function of glutathione-S-transferase (GST α) (30) or endoplasmic transport for conjugated bilirubin (31, 32). Another theory deals with an 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.

Conclusion

Our work 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), by identification and functional analysis of the regulatory regions of the *ATP8B1* gene, description of a yet unrecognised mixed type digenic hyperbilirubinemia and by exclusion of 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.

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 complexity of *ATP8B1* gene regulation, identifying new *ATP8B1* mRNA isoforms that differ in 5'UTRs 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 an allelic variant of DJS.

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List of publications related to PhD thesis

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.
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2. *Hrebicek M, Jirasek T, Hartmannova H, Noskova L, Stranecky V, Ivanek R, Kmoch S, Cebecauerova D et al.*
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3. *Kotalová R., Cebecauerová D., Knisely A.S., Hřebíček M., Jirsa M.*
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5. *Jirsa M., Cebecauerová D., Budišová L., Chuzhanova N., Hřebíček M. et al.*
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List of publications not related to PhD thesis

None