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Autoreferát disertační práce/Ph.D. Thesis – Short Report

Sekreční dráha bilirubinu a její poruchy

Bilirubin secretory pathway and its disorders

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Abstrakt

Identifikace a funkční charakterizace řady přenašečových systémů na sinusoidální a kanalikulární membráně hepatocytů významně přispěly k objasnění molekulární podstaty dědičných hyperbilirubinémií. Porucha regulace hepatobiliárních transportních systémů se rovněž podílí na vzniku žloutenky u mnoha získaných jaterních chorob. Předkládaná práce rozšiřuje současné znalosti metabolické dráhy degradace hemu s důrazem na nové poznatky o mechanismu transportu bilirubinu v hepatocytech, vyplývající z objasnění molekulární podstaty Rotorova syndromu.

První předkládaná práce se zabývá antioxidačním působením bilirubinu v jaterní tkáni u modelu obstrukční žloutenky. Ve druhé práci jsme v rámci charakterizace nemocných s dědičnými formami konjugované hyperbilirubinémie zachytili několik nových mutací v genu *ABCC2*, jehož deficit podmiňuje Dubin-Johnsonův syndrom. V klíčové třetí práci o Rotorově syndromu jsme ukázali, že příčinou hyperbilirubinémie Rotorova typu je digenní porucha jaterního vychytávání konjugovaného bilirubinu podmíněná deficitem OATP1B1 a OATP1B3. Ukazuje se, že přímo do žluče je secernována pouze část bilirubinu konjugovaného v hepatocytech. Zbytek je nejprve vyloučen do krve prostřednictvím transportéru MRP3 a teprve následně vychytán zpět sinusoidálními transportéry OATP1B1 a OATP1B3. Ve čtvrté práci jsme potvrdili, že při konjugované hyperbilirubinémii provázející pokročilá stádia cholestatických onemocnění jater dochází ke snížení exprese rotorovských transportérů. Dále jsme zjistili, že exprese proteinů OATP1B inverzně koreluje s hladinou konjugovaného i celkového bilirubinu v séru. Je tedy velmi pravděpodobné, že pokles exprese až absence OATP1B1 a OATP1B3 je vedle zvýšení exprese MRP3 dalším mechanismem přispívajícím ke zvýšení konjugované složky hyperbilirubinémie v terminálních stádiích jaterních chorob provázených zejména obstrukčním typem cholestázy.

Klíčová slova: Bilirubin, hyperbilirubinémie, žloutenka, cholestáza

Abstract

Identification and functional characterization of numerous transport systems at the sinusoidal and canalicular membrane of hepatocytes have significantly expanded our understanding of bilirubin metabolism and contributed to elucidation of molecular basis of hereditary jaundice. Moreover, dysregulation of hepatobiliary transport systems could explain jaundice in many acquired liver disorders. This thesis is focused on the new aspects of bilirubin handling in hepatocytes based on elucidation of the molecular basis of Rotor syndrome.

The first study is focused on the antioxidative properties of bilirubin in liver tissue in a model of obstructive cholestasis. In the second part of the thesis we present several novel mutations in *ABCC2,* the gene associated with Dubin-Johnson syndrome, identified in patients selected for the Rotor locus mapping study. In the key third study concerned with Rotor syndrome we demonstrated that biallelic inactivating mutations causing complete absence of transport proteins OATP1B1 and OATP1B3 result in disruption of hepatic reuptake of bilirubin, which is the molecular basis of Rotor-type jaundice. These results indicate that apart from secretion of conjugated bilirubin into bile, a significant fraction of bilirubin glucuronide is secreted via MRP3 into sinusoidal blood and subsequently reuptaken by sinusoidal transporters OATP1B1 and OATP1B3. We further confirmed that Rotor proteins are down-regulated in advanced stages of cholestatic liver disorders. We demonstrated that OATP1Bs expression inversely correlates with serum levels of conjugated and total bilirubin. We suppose that aside from increased MRP3 expression, down-regulation of OATP1B1 and OATP1B3 contributes to conjugated hyperbilirubinemia in advanced liver diseases with predominantly obstructive type of cholestasis.

Keywords: Bilirubin, hyperbilirubinemia, jaundice, cholestasis

Content

1 INTRODUCTION

1.1 Heme degradation pathway

Bilirubin is the end product of heme breakdown. About 80% of bilirubin originates from degradation of erythrocyte haemoglobin in the reticuloendothelial system (RES), the remaining 20% comes from inefficient erythropoiesis in bone marrow and degradation of other heme proteins (Berk P.D. et al., 1979). Water insoluble, unconjugated bilirubin (UCB) bound to albumin is transported to the liver where it is removed from the plasma. Within the cytoplasm of hepatocytes, bilirubin is transported to endoplasmic reticulum (ER) where conjugation with glucuronic acid takes place. The reaction is catalysed by the enzyme uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), a member of an enzyme family in the ER and nuclear envelope of hepatocytes (Bosma P.J. et al., 1994). Conjugated bilirubin is transported into bile against a concentration gradient. The energy for this unidirectional active transport is derived from adenosine triphosphate (ATP) hydrolysis by the canalicular ATP-binding cassette (ABC) protein identified as the multidrug resistance-associated protein MRP2/cMOAT (Nishida T. et al., 1992).

As conjugated bilirubin reaches the terminal ileum and the large intestine, the molecules of glucuronic acid are cleaved by bacterial β-glucuronidases and the pigment is subsequently reduced by the fecal flora to a group of colorless tetrapyrrolic compounds called urobilinogens. Urobilinogen can be metabolized to stercobilinogen and stercobilin. Most of the urobilinogens are oxidized to colored urobilins and excreted in the feces. A small fraction of urobilinogens is re-absorbed and re-excreted through the liver to constitute the enterohepatic urobilinogen cycle. Some urobilinogen is re-absorbed and subsequently excreted in the urine along with urobilin (Fahmy K. et al., 1972).

1.2 Transporters of biliary lipids and pigments in hepatocytes

To secrete bile and excrete various metabolites of endogenous or exogenous origin, liver cells must transport bilirubin, bile salts, phospholipids, and other substrates from portal blood to bile against a concentration gradient. Hepatocytes express polarized transport systems at the basolateral (sinusoidal) and apical (canalicular) plasma membrane domains.

1.2.1 Basolateral transport proteins

Basolateral transport systems involved in blood secretion and liver uptake of small molecules belong to the solute carrier (SLC) superfamily and MRP subfamily of the ATP-binding cassette (ABC) transporter superfamily, respectively. SLC proteins include the Na^+-

dependent transporter for the uptake of bile salts (NTCP; gene symbol *SLC10A1*), transporters for amphiphilic substrates such as organic anion-transporting polypeptides (OATPs; gene family *SLCO*, former *SLC21A*) and organic cation transporters (OCTs; gene family *SLC22A*) (Hagenbuch B. and Meier P.J., 1996, Roth M. et al., 2012). In general, ABC transporters are responsible for efflux of substrates, while SLC transporters mediate uptake of substrates into cells.

Organic anion-transporting polypeptides (OATPs in humans, Oatps in rodents) are multispecific ATP- and Na^+-independent uptake transporters expressed in numerous epithelial cells throughout the body, transporting predominantly large and hydrophobic organic anions, selected organic cations, anti-HIV drugs, statins, and wide range of chemotherapeutics (Roth M. et al., 2012).

OATP1B1 (gene *SLCO1B1*) and OATP1B3 (gene *SLCO1B3*), the major OATP1Bs in humans, are highly homologous proteins with similar genomic organization into 15 exons. Expression of *SLCO1B1* and *SLCO1B3* is restricted to human hepatocytes and the corresponding protein products are localized to the basolateral (sinusoidal) membrane (König J. et al., 2000a, König J. et al., 2000b). Several polymorphisms in *SLCO1B1* and *SLCO1B3* have been identified. The OATP1B1 rs2306283 polymorphism p.N130D is associated with development of severe hyperbilirubinemia in neonates (Büyükkale G. et al., 2011), the OATP1B1 rs4149056 polymorphism p.V174A with higher serum bilirubin levels in healthy adults (Ieiri I. et al., 2004), and two non-coding variants in *SLCO1B3* may contribute to idiopathic mild unconjugated hyperbilirubinemia (Sanna S. et al., 2009). These data, together with the observations of other groups (Cui Y. et al., 2001, Briz O. et al. 2003), support the concept that OATP1Bs are involved in liver uptake of unconjugated bilirubin.

1.2.2 Canalicular transport proteins

Most canalicular transporters for biliary lipids and pigments belong to the ATP-binding cassette (ABC) transporter superfamily, utilizing the energy of ATP hydrolysis to drive the transport of various molecules across cell membranes and non-transport-related processes such as translation of RNA and DNA repair (Dean M. et al., 2001). With respect to bile formation, the most important canalicular transporters include bile salt export pump (BSEP, gene *ABCB11*), responsible for transport of predominantly monovalent conjugated bile acids (Gerloff T. et al., 1998), and multidrug resistance-associated protein MRP2/cMOAT (gene *ABCC2*), transporting conjugates of endogenous and xenobiotic

compounds, including bilirubin glucuronates and glutathione (Nishida T. et al., 1992, Paulusma C.C. et al., 1996, Keppler D. et al. 1997).

1.3 Pathophysiology of hyperbilirubinemia and jaundice

Normal serum bilirubin concentration in adults is less than 17 μmol/l. Hyperbilirubinemia denotes increased serum bilirubin levels above 20 μmol/l. Jaundice (icterus), a yellow discoloration of tissues, namely the sclera of the eyes and skin, is the clinical manifestation of hyperbilirubinemia noticeable at serum bilirubin levels exceeding 30-50 μmol/l. Hyperbilirubinemia, either of conjugated or unconjugated type, may exist as an isolated pathologic finding. However, more frequently, hyperbilirubinemia and jaundice present as a part of cholestasis, characterized by a decrease in bile flow due to impaired secretion by hepatocytes or to obstruction of bile flow through intra- or extrahepatic bile ducts. Cholestasis is associated with retention of bile salts, free cholesterol, biliary pigments, phospholipids, and other constituents normally excreted into bile.

Depending on the predominant type of bile pigment in the plasma, hyperbilirubinemia is classified into three major categories: unconjugated (indirect) hyperbilirubinemia – premicrosomal type, unconjugated (direct) hyperbilirubinemia – postmicrosomal type, and mixed hyperbilirubinemia (Vítek L., 2009).

1.3.1 Bilirubin handling proteins in cholestasis

Up- and down-regulation of a broad range of transport systems involved in bile formation can explain impaired liver uptake and excretion of the biliary constituents resulting in cholestasis and jaundice which accompanies some hereditary and many common acquired liver disorders. A general pattern of response to cholestatic liver injury comprises down-regulation of the basolateral membrane bound transporters NTCP and OATPs (Kojima H. et al., 2003, Geier A. et al., 2007). Expression of several canalicular export pumps is relatively unaffected (BSEP) or even up-regulated (MDR1). Decreased expression of MRP2 in sepsis or in obstructive cholestasis is followed by up-regulation of several MRP homologues (particularly MRP3) at the basolateral membrane of hepatocytes (Geier A. et al., 2007, Nies A.T. and Keppler D, 2007). Most of these changes are believed to represent compensatory mechanisms that may help prevent accumulation of potentially toxic bile components and other substrates in the liver (Kullak-Ublick G.A. et al, 2004).

1.4 Bilirubin toxicity and antioxidant properties

Bilirubin is generally regarded as a potentially cytotoxic waste product. Patients with profound unconjugated hyperbilirubinemia are at risk for the development of bilirubin-induced neurological dysfunction (BIND) and kernicterus (Shapiro S.M., 2010). Nevertheless, recent data have indicated the potent antioxidant properties of mild or moderately elevated serum bilirubin levels with substantial positive clinical consequences, especially their protective effects on atherogenesis and cancerogenesis (Stocker R. et al., 1987, Ollinger R. et al., 2007, Vítek L. and Schwertner H.A., 2008).

Apart from antioxidative properties, antiinflammatory, antiproliferative, cytostatic and pro-apoptotic effects of bilirubin are well-known (Keshavan P. et al., 2004, Ollinger R. et al., 2007).

1.5 Inherited forms of hyperbilirubinemia

1.5.1 Inherited forms of predominantly unconjugated hyperbilirubinemia

Three types of inherited, predominantly unconjugated hyperbilirubinemia with different levels of UGT1A1 activity are recognized: Crigler-Najjar syndrome type I, type II and Gilbert syndrome.

Crigler-Najjar syndrome type I (CN1, MIM#218800), the most deleterious form, is characterized by complete or almost complete absence of UGT1A1 enzyme activity with severe jaundice (Crigler J.F. Jr. and Najjar V. A., 1952, Ritter J.K. et al., 1992). Icterus occurring shortly after birth is complicated by bilirubin encephalopathy (kernicterus).

Crigler-Najjar syndrome type II (Arias syndrome, CN2, MIM#606785) is characterized by reduced UGT1A1 enzyme activity with a moderate degree of non-haemolytic jaundice (Arias I.M., 1962). Bilirubin levels do not exceed 350 μmol/l and CN2 is only rarely complicated by kernicterus (Gollan J.L. et al., 1975).

Gilbert syndrome (GS, MIM#143500) is characterized by fluctuating mild, unconjugated non-haemolytic hyperbilirubinemia < 85 μmol/l, usually diagnosed around puberty, and aggravated by intercurrent illness, stress, fasting or after administration of certain drugs (Gilbert A. and Lereboullet P., 1901). GS is characterized by reduced levels of UGT1A1 activity to about 25-30% caused by homozygous, compound heterozygous, or heterozygous mutations in the *UGT1A1* with autosomal recessive transmission (Black M. and Billing B.H., 1969).

1.5.2 Inherited forms of predominantly conjugated hyperbilirubinemia

Two types of hereditary conjugated jaundice are known as Dubin-Johnson syndrome and Rotor syndrome. Both are characterized by the presence of mixed, predominantly conjugated hyperbilirubinemia, with conjugated bilirubin more than 50% of total bilirubin.

Dubin-Johnson syndrome (DJS, MIM#237500), a benign autosomal recessive disorder is characterized by fluctuating mild, predominantly conjugated hyperbilirubinemia, with typical manifestation in adolescence or young adulthood (Dubin I.N. and Johnson F.B., 1954, Sprinz H. and Nelson R.S., 1954). Urine excretion of total coproporphyrin in 24 hours is normal, but 80% are represented by coproporphyrin I. Biliary excretion of anionic dyes including BSP, indocyanine green and cholescintigraphy radiotracers is delayed with absent or delayed filling of the gallbladder (Shani M. et al., 1970). Liver histology in DJS shows an accumulation of distinctive melanin-like lysosomal pigment in an otherwise normal liver that gives the organ a characteristic dark pink or even black colour (Swartz H.M. et al., 1987). The molecular mechanism in DJS is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation in *ABCC2* on chromosome 10q24 (Paulusma C.C. et al., 1996, Toh S. et al., 1999).

A rare type of hereditary mixed hyperbilirubinemia resulting from simultaneous occurence of mutations characteristic for DJS and GS was classified as ***dual hereditary jaundice*** (Cebecauerova D. et al., 2005).

Rotor syndrome (RS, MIM #237450) is a rare familial disorder with autosomal recessive transmission, which is characterized by mild, predominantly conjugated hyperbilirubinemia with delayed excretion of anionic dyes without re-increase of their concentration (Rotor B. et al., 1948). Total urinary coproporphyrin excretion is significantly increased and the proportion of coproporphyrin I in urine is approximately 65% of the total in homozygotes and 43% in heterozygotes (Wolkoff A.W. et al., 1976, Wolpert E. et al., 1977). Apart from predominantly conjugated hyperbilirubinemia, clinical findings and liver tests are normal. Molecular genetic analysis excluded pathogenic mutations in *ABCC2* and confirmed that RS is not allelic variant of DJS (Hrebicek M. et al., 2007).

2 SPECIFIC AIMS

This thesis is focused on the molecular aspects of bilirubin transport and inherited forms of hyperbilirubinemia with emphasis on predominantly conjugated type of hereditary hyperbilirubinemia syndromes.

Our objectives were:

1. To evaluate the role of bilirubin tissue concentration on mediating oxidative stress in cholestatic liver
2. To characterize at the molecular level subjects with predominantly conjugated type of hereditary jaundice
3. To determine the molecular basis of predominantly conjugated Rotor-type jaundice
4. To assess the role of Rotor proteins OATP1B1 and OATP1B3 in pathogenesis of jaundice

3 METHODS

3.1 General methods

Nucleic acid isolation from human cells and tissues

Restriction analysis of DNA

Polymerase chain reaction

DNA electrophoresis on agarose and polyacrylamide gel

Molecular cloning techniques

Direct sequencing of genomic DNA

Histochemistry and immunohistochemistry on frozen and paraffin sections

Electron microscopy

Experimental work with small laboratory animals

Statistical methods

3.2 Specific methods

3.2.1 Methods related to evaluation the role of bilirubin and bile acids on mediating oxidative stress

Animal and experimental protocol. Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and biliary trees were exposed through midline abdominal incisions. Microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed. Sham-operated rats underwent the same procedure without bile duct resection and ligation.

Histopathological analysis. Left lateral lobes of livers were fixed overnight in 10% buffered formalin (pH 7.4) at 4°C followed by a standard procedure for paraffin embedding. Serial sections (6 μm thick) were cut and stained with H&E, Shikata's orcein method, and elastic-van Gieson stain.

3.2.2 Methods related to characterization of subjects with predominantly conjugated type of hereditary jaundice

Mutational analysis of ABCC2. Written informed consent was obtained from the patients before their genetic examination. *ABCC2* was analysed by direct sequencing of genomic DNA extracted from peripheral leucocytes. All 32 exons, with the adjacent parts of the intronic sequences, were amplified by PCR using the intronic oligonucleotide primers. Amplified fragments were gel-purified, extracted from the gel with QIA quick spin columns (Qiagene, Hilden, Germany), and sequenced on a Genetic Analyzer ABI 3130 (Life Technologies, Prague, Czech Republic). The obtained sequence was compared with the reference sequences GenBank NM_000392 (mRNA) and NT_030059 (genomic DNA). Exon 18 with suspected deletion (c.2360_2366delCCCTGTC) was cloned into a plasmid vector pCR4.1-TOPO (Invitrogen, Carlsbad, CA), and the wild-type and mutated alleles were sequenced separately. Presence of the second mutation in DJS subject (see Chapter 4.2) was confirmed by PCR - restriction fragment - lenght polymorphism analysis (*PCR-Bsh1236*I *RFLP).*

The pathogenicity of the sequence variants in *ABCC2* detected in the probands was predicted by the GeneSplicer software (http://ccb.jhu.edu/software/genesplicer/), Pmut software (http://mmb2.pcb.ub.es:8080/PMut/) and/or PredictSNP 1.0 software (http://loschmidt.chemi.muni.cz/predictsnp/).

Histopathological methods and ultrastructural analysis of liver in DJS subject. Sections of the formalin-fixed paraffin-embedded liver tissue cut at 4-6 μm were stained with H&E,

periodic acid-Schiff with diastase (PAS-D), Schmorl´s and van Gieson´s method. Special stainings (Gömöri, silver ammonium complex - Masson´s and Perls Prussian blue method) for pigment characterization were added.

For immunohistochemical analysis, 4-6 μm - thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2III-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualisation and counterstaining with Harris´s hematoxylin was performed.

Ultrastructural analysis was performed on formalin-fixed liver sample osmicated, dehydrated in ascending ethanol solutions and embedded into Epon-Araldite mixture. Ultrathin sections were double stained with uranyl acetate and lead nitrate and then examined under a JEM 1200 EX electron microscope.

3.2.3 Methods related to Rotor study

Histopathological analysis. Sections of archival paraffin-embedded human liver tissue (formalin or Carnoy solution fixative; 4–6 μm thick) were stained with H&E, elastic-van Gieson stain and PAS-D techniques.

For OATP1B1 and OATP1B3 immunostaining, deparaffinized sections were treated in 10 mM sodium citrate buffer, pH 6.0, for 30 min at 96°C, and incubated with primary mouse anti-OATP1B antibody (clone MDQ; ab15442, Abcam, Cambridge, UK), 1:100 dilution, overnight at 4°C. Bound antibody was visualized with horseradish peroxidase/diaminobenzidine (EnVision), with hematoxylin counterstaining.

For MRP2 immunostaining, 4-6 μm - thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2III-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualisation and counterstaining with Harris´s hematoxylin was performed.

3.2.4 Methods related to evaluation the role of OATP1B1 and OATP1B3 transporters in pathogenesis of jaundice

Mutational analysis of UGT1A1 and SLCO1B1. *UGT1A1* TATA-box promoter polymorphism rs8175347 and the *SLCO1B1* c.521T>C (p.V174A) coding polymorphism rs4149056 were genotyped by direct sequencing of genomic DNA extracted from peripheral leucocytes on the Applied Biosystems ABI 3130 genetic analyzer (Life Technologies, Prague, Czech Republic).

Histopathological analysis. The 4 μm thick paraffin sections of formalin-fixed mouse and human liver tissue were pretreated by incubation with Proteinase K (Dako, Glostrup,

Denmark) or in citrate buffer - pH 6.0 (Dako), Tris/EDTA buffer pH 8.0 (Leica, Wetzlar, Germany), Tris/EDTA buffer pH 9.0 (Dako) and High pH buffer (Dako). Sections without pretreatment were also used in parallel. Subsequent incubations with primary antibodies recognizing either N- or C- terminus of OATP1B1 and/or OATP1B3 (dilution 1:50 and 1:100) were done overnight at +4° C. For detection of primary antibodies a two-step (Dako, Histofine) or a three-step (Vector, Laboratories, Burlingame, CA) visualization system was used. Counterstaining with Harris's hematoxylin was performed at the end.

To minimize the reactivity of the secondary anti-mouse antibody with endogenous immunoglobulin in the mouse tissue, sections of mouse livers were stained with the Dako ARK^{TM} (Animal Research Kit) Peroxidase (Dako).

Statistical analysis. Results were expressed as the mean ± SD. To calculate the statistical significance of the differences between the groups, the Mann-Whitney test was used. The relations between the parameters were estimated by the nonparametric Spearman´s correlation coefficient. An exponential model was used for significant correlations. Two-sided $p<0.05$ was considered statistically significant.

Detailed description of the methods listed above, all performed by the author, is a part of a methodology section of the articles enclosed to this thesis (Enclosures No. 3-6).

4 RESULTS

4.1 The role of bilirubin tissue concentration on mediating oxidative stress in the cholestatic liver

The role of bilirubin and bile acids on mediating oxidative stress in Wistar and hyperbilirubinemic Gunn rats following bile duct ligation (BDL) was evaluated in this study. The results demonstrate that:

a) bilirubin increases peroxyl radical scavenging capacity in plasma, but not in liver homogenates in BDL rats
b) intracellular bilirubin levels in hepatocytes are relatively decreased compared to plasma in BDL animals
c) bilirubin production is decreased and lipid peroxidation is increased after BDL
d) taurocholic acid increases lipid peroxidation in the liver homogenates and decreases intracellular bilirubin levels in HepG2-rNtcp cells.

To induce cell injury in hepatocytes during cholestasis, a combination of increased ROS formation, especially due to accumulation of bile acids, and impairment of the antioxidant defense systems is necessary. The results of this study indicate that bilirubin levels and the antioxidant capacity in plasma are increased in obstructive cholestasis; however, bilirubin in the liver tissue is relatively decreased compared to plasma. We conclude that the increase in intracellular bile acids/bilirubin ratio in obstructive cholestasis may be implicated in the oxidative stress-mediated cholestatic liver injury.

4.2 Characterization of subjects with predominantly conjugated type of hereditary jaundice at the molecular level

We analyzed six individuals with long-term predominantly conjugated type of hyperbilirubinemia. Molecular genetic alterations in *ABCC2* identified in the analyzed probands are summarized in Tab. 1.

Tab. 1: **Molecular genetic alterations in *ABCC2* identified in six analyzed probands**

Proband	DNA alteration	Protein alteration	Pathogenicity	MAF
1	Heterozygous **c.2360_2366delCCCTGTC**	p.Pro787LeufsX7	Pathogenic	
	Heterozygous **c.3258+1G>A**	abnormal splicing	Pathogenic	
2	Heterozygous c.1249G/A	p.Val417Ile	SNP rs2273697	0.174
	Heterozygous c.1446C/G	no	SNP rs113646094	0.003
	Heterozygous **c.2213C>G**	p.Ala738Gly	Likely pathogenic	
	Heterozygous **c.2310C>G**	p.Ser770Arg	Likely pathogenic	
3	Homozygous c.116G/A	p.Tyr39Phe	SNP rs927344	0.004
	Heterozygous c.1249C/G	p.Val417Ile	SNP rs2273697	0.174
4	Heterozygous c.2009T/C	p.Ile670Thr	SNPrs17222632	0.005
	Heterozygous **c.2741G>A**	p.Ser914Asn	Uncertain	
	Homozygous c.3563T/A	p.Glu1188Val	SNP rs17222723	0.043
	Heterozygous c.4290G/T	p.Val1430Val	SNP rs1137968	0.043
	Heterozygous c.4544G/A	p.Cys1515Tyr	SNP rs8187710	0.070
5	Heterozygous c.1249G/A	p.Val417Ile	SNP rs2273697	0.174
6	Heterozygous c.3972C/T	no	SNP rs3740066	0.288

MAF – minor allele frequency in GenBank dbSNP

Mutation analysis of *ABCC2* (all subjects) and *UGT1A1* gene promoter (proband 2) clarified the cause of predominantly conjugated hyperbilirubinemia in two of the six analyzed index subjects: Dubin-Johnson syndrome (DJS) caused by two novel pathogenic mutations was identified in Proband 1. Hereditary hyperbilirubinemia in Proband 2 is likely caused by simultaneous mutations in both *ABCC2* and *UGT1A1* and may correspond to dual hereditary jaundice (Cebecauerova D. et al., 2005).

DJS was not confirmed in the other four subjects and further investigations were necessary to clarify the cause of hyperbilirubinemia (see Chapter 4.3).

4.3 Molecular basis of predominantly conjugated Rotor-type jaundice

The study has animal and human part.

The animal studies held by the group of Dr. Alfred Schinkel, The Netherlands Cancer Institute, Amsterdam showed that Abcc3 mediates bilirubin glucuronide excretion from the liver to sinusoidal blood and Oatp1a/b transporters mediate its hepatic reuptake. Experiments with *SLCO1B1* and *SLCO1B3* transgenic mice with liver specific expression of OATP1B1

and OATP1B3 generated on the background of the *Slco1a/b* deficiency indicate that either of the human OATP1B proteins reuptakes conjugated bilirubin from plasma to hepatocytes.

The parallel human mapping study conducted by the Czech group in 11 Rotor subjects including our Probands 4, 5 and 6 with unexplained predominantly conjugated hyperbilirubinemia (see Chapter 4.2) and in other three male subjects (1 Dutchman, 2 Turks) analyzed during the years 2012 – 2013 demonstrated that biallelic inactivating mutations of the human OATP genes *SLCO1B1* and *SLCO1B3* resulting in simultaneous and complete OATP1B1 and OATP1B3 deficiency may explain predominantly conjugated hyperbilirubinemia of Rotor-type.

All RS subjects were homozygous for biallelic inactivating mutations in both *SLCO1B1* and *SLCO1B3.* Three haplotypes were identified: a biallelic nonsense mutation in *SLCO1B1* and a biallelic deletion of exon 12 in *SLCO1B3;* a biallelic whole-gene deletion spanning both *SLCO1B1* and *SLCO1B3*; a nonsense mutation in *SLCO1B1* and a biallelic splice site mutation in *SLCO1B3*.

Additionally, we completed mutation analysis of *SLCO1B1* and *SLCO1B3* in Proband 3 (Chapter 4.2) with still unexplained predominantly conjugated hyperbilirubinemia. Since only seven non-pathogenic sequence variations were disclosed, RS was not confirmed in this subject.

4.4 The role of Rotor proteins OATP1B1 and OATP1B3 in the pathogenesis of jaundice

In the last study we correlated immunohistochemical expression of Rotor proteins in formalin-fixed paraffin-embedded liver tissue with serum bilirubin levels in advanced stages of common liver diseases.

First, immunoreactivity of five antibodies directed against human OATP1B1 and/or OATP1B3 was tested on frozen and formalin-fixed paraffin-embedded liver tissue of mouse strains transgenic for *SLCO1B1* or *SLCO1B3* and on human specimens.

The most specific detection of OATP1B3 was achieved with the anti OATP1B3 H-52 (sc-98981) antibody. OATP1B1 was specifically recognized with the ESL (ab15441) anti-OATP1B1 antibody, but only in frozen sections. The MDQ (ab15442) anti-OATP1B1 antibody cross-reacted with both OATP1B proteins in frozen and formalin-fixed liver tissue of the transgenic mouse strains.

In the second part of the study, the proportion of hepatocytes expressing OATP1B1/3 was semi-quantitatively assessed with the MDQ antibody in formalin-fixed liver samples obtained from the patients with end-stage hepatocellular (n=21) and biliary diseases (n=31). The immunohistochemical OATP1Bs expression was correlated with serum bilirubin levels in these patients. *UGT1A1* promoter TATA-box and *SLCO1B1* rs4149056 genotyping was performed to rule out individuals genetically predisposed to hyperbilirubinemia.

Expression of the OATP1B proteins was decreased in advanced liver diseases and inversely correlated with conjugated and total serum bilirubin levels. The reduction was more pronounced in the group of primary biliary diseases (1.9±1.1 vs. 2.7±0.6; p=0.009).

5 CONCLUSIONS

1. We contributed to clarification of the role of bilirubin in pathogenesis of oxidative stress in a model of obstructive jaundice.
2. We explained the cause of predominantly conjugated hyperbilirubinemia in five of the six analyzed index subjects:
   - DJS caused by two novel pathogenic mutations was identified in one proband
   - simultaneous mutations in *ABCC2* and *UGT1A1* promoter likely associated with dual hereditary jaundice were detected in one proband
   - mutations associated with Rotor type of jaundice were detected in three probands

   In one subject mutations associated with either RS or DJS were not demonstrated by mutation analysis of *ABCC2, SLCO1B1* and *SLCO1B3*.
3. We brought new insights in the role of transport proteins OATP1B1 and OATP1B3 in the heme degradation pathway and disclosed still unknown hepatic cycle of conjugated bilirubin and many other substrates. Additionally, we demonstrated that simultaneous inactivating mutations in *SLCO1B1* and *SLCO1B3* with complete absence of basolateral transport proteins OATP1B1 and OATP1B3 resulting in disturbed uptake of conjugated bilirubin by hepatocytes represent molecular basis of Rotor syndrome.
4. We confirmed that decreased expression of OATP1B1 and OATP1B3 in hepatocytes may contribute to the pathogenesis of jaundice accompanying advanced stages of acquired liver diseases, particularly with obstructive type of cholestasis.

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7 LIST OF AUTHOR´S PUBLICATIONS

7.1 Publications in extenso related to the topic of the Ph.D. thesis

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